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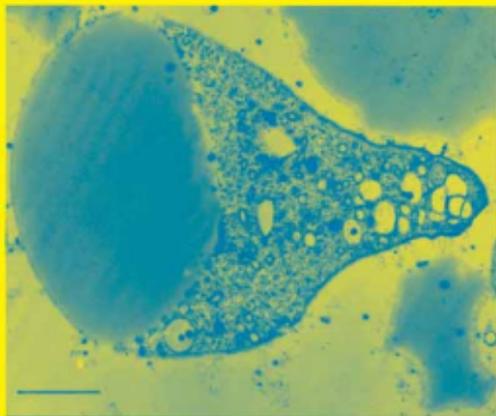
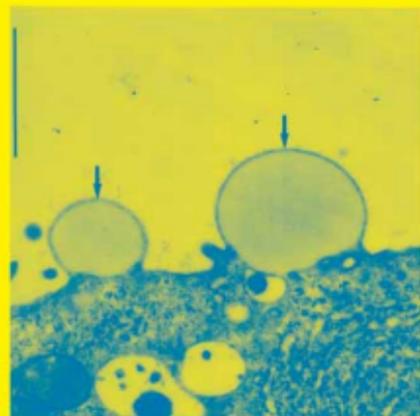
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# VOLUME 2

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# LIPIDS

THIRD EDITION



**EDITED BY P.F. FOX  
AND P.L.H. McSWEENEY**

# **Advanced Dairy Chemistry**

# **Advanced Dairy Chemistry**

## **Volume 2 Lipids**

Third Edition

Edited by

**P. F. FOX and P. L. H. McSWEENEY**

*University College Cork, Ireland*

 **Springer**

Library of Congress Control Number: 2005928170

ISBN-10: 0-387-26364-0 e-ISBN: 0-387-28813-9

ISBN-13: 978-0387-26364-9

Printed on acid-free paper.

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Printed in the United States of America (SPI/SBA)

10 9 8 7 6 5 4 3 2 1

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## Preface to the Third Edition

*Advanced Dairy Chemistry 2: Lipids* is the second volume of the third edition of the series on advanced topics in Dairy Chemistry, which started in 1982 with the publication of *Developments in Dairy Chemistry*. The first volume, on milk proteins, of the third edition of *Advanced Dairy Chemistry* was published in 2003. This series of volumes is intended to be a coordinated and authoritative treatise on Dairy Chemistry. In the decade since the second edition of this volume was published (1995), there have been considerable advances in the study of milk lipids, which are reflected in changes to this book.

Most topics included in the second edition are retained in the current edition, which has been updated and considerably expanded from 10 to 22 chapters. For various reasons, the authors of many chapters have been changed and hence, in effect, are new chapters, at least the topic is viewed from a different perspective.

The new chapters cover the following subjects: Biosynthesis and nutritional significance of conjugated linoleic acid, which has assumed major significance during the past decade; Formation and biological significance of oxysterols; The milk fat globule membrane as a source of nutritionally and technologically significant products; Physical, chemical and enzymatic modification of milk fat; Significance of fat in dairy products: creams, cheese, ice cream, milk powders and infant formulae; Analytical methods: chromatographic, spectroscopic, ultrasound and physical methods.

Like its predecessor, this book is intended for academics, researchers at universities and industry, and senior students; each chapter is referenced extensively.

We wish to thank sincerely the 37 contributors to the 22 chapters of this volume, whose cooperation made our task as editors a pleasure. The generous assistance of Ms. Anne Cahalane is gratefully acknowledged.

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# Preface to the Second Edition

*Advanced Dairy Chemistry* can be regarded as the second edition of *Developments in Dairy Chemistry*. The first volume in the series, on Milk Proteins, was published in 1992; this, the second volume, is devoted to Milk Lipids. Considerable progress has been made in several aspects of milk lipids during the past 11 years which is reflected in revised versions of seven of the eight chapters included in *Developments in Dairy Chemistry* –2, most of them by the same authors. The theme of one chapter has been changed from physical properties and modification of milk fat to the crystallization of milk fat. Two new chapters have been added, i.e. chemistry and technology aspects of low-fat spreads and the significance of fat in consumer perception of food quality, which reflect the continuing consumer awareness of a healthy diet. Low-fat spreads have become increasingly significant during the past decade and are now the major type of spread in many countries. However, reducing the fat content of foods generally results in a concomitant decrease in the organoleptic quality of the food; consumer attitudes to reduced-fat dairy products are discussed in one of the new chapters.

Like its predecessor, the book is intended for lecturers, senior students and research personnel and each chapter is extensively referenced.

I would like to thank all the authors who contributed to this book and whose cooperation made my task as editor a pleasure.

**P. F. Fox**

# Preface to the First Edition

Many of the desirable flavour and textural attributes of dairy products are due to their lipid components; consequently, milk lipids have, traditionally, been highly valued, in fact to the exclusion of other milk components in many cases. Today, milk is a major source of dietary lipids in western diets and although consumption of milk fat in the form of butter has declined in some countries, this has been offset in many cases by increasing consumption of cheese and fermented liquid dairy products.

This text on milk lipids is the second in a series entitled *Developments in Dairy Chemistry*, the first being devoted to milk proteins. The series is produced as a co-ordinated treatise on dairy chemistry with the objective of providing an authoritative reference source for lecturers, researchers and advanced students. The biosynthesis, chemical, physical and nutritional properties of milk lipids have been reviewed in eight chapters by world experts. However, space does not permit consideration of the more product-related aspects of milk lipids which play major functional roles in several dairy products, especially cheese, dehydrated milks and butter.

Arising from the mechanism of fatty acid biosynthesis and export of fat globules from the secretory cells, the fat of ruminant milks is particularly complex, containing members of all the major lipid classes and as many as 400 distinct fatty acids. The composition and structure of the lipids of bovine milk are described in Chapter 1, with limited comparison with non-bovine milk fats. Since the fatty acid profile of milk fat, especially in monogastric animals, may be modified by diet and other environmental factors, the biosynthesis of milk lipids is reviewed in Chapter 2 with the objective of indicating means by which the fatty acid profile, and hence the functional properties of the lipids, might be modified. Lipids in foods are normally present as an emulsion, stabilized by a layer of protein adsorbed at the oil-water interface. The fat in milk and cream exists as an oil-in-water emulsion with a unique stabilizing lipoprotein membrane, referred to as the milk fat globule membrane (MFGM). The inner layers of the MFGM are formed within the secretory cell and are relatively stable; however, the outer layers, which are acquired as the fat globule is exported through the apical membrane of the secretory cells, are unstable. Damage to the MFGM leads to chemical and physical instability of the fat phase in milk and hence the

structure of the membrane has been the subject of considerable research, the results of which are reviewed in Chapter 3.

Lipids strongly influence, for good or evil, the flavour and texture of foods, especially high-fat products such as butter. The influence of various colloidal features of milk fat on the properties of milk and cream is considered in Chapter 4, while the crystallization of milk fat and how this may be controlled, modified and measured are reviewed in Chapter 5. Unfortunately, lipids are subject to chemical and enzymatic alterations which can cause flavour defects referred to as oxidative and hydrolytic rancidity, respectively. The storage stability of high-fat foods, especially mildly flavoured foods like milk, cream and butter, is strongly influenced by these changes which have been reviewed in Chapters 6 and 7.

Dietary lipids play many diverse nutritional roles, some of which are essential. However, dietary lipids, especially saturated lipids of animal origin, have been the subject of much controversy in recent years, particularly in regard to their possible role in atherosclerosis. Various aspects of the nutritional significance of lipids are discussed in Chapter 8.

Finally, I wish to thank sincerely the 14 authors who have contributed to this text and whose co-operation has made my task as editor a pleasure.

**P. F. Fox**

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# Composition and Structure of Bovine Milk Lipids

A.K.H. MacGibbon and M.W. Taylor

## 1.1. Introduction

The lipids in bovine milk are present in microscopic globules as an oil-in-water emulsion. The primary purpose of these lipids is to provide a source of energy to the newborn calf. Both the fat content of the milk and the fatty acid composition of the lipids can vary considerably as a result of changes in factors like breed of cow, diet and stage of lactation. The fat content can vary from about 3.0 to 6.0%, but typically is in the range 3.5 to 4.7%. Changes in the composition of the fatty acids (e.g., 16:0 and 18:1) can be quite marked and can lead to changes in physical properties of the fat. These changes make comparison difficult between different samples of milk fat, and ideally comparisons should be made between cows in mid-lactation and fed on similar diets. From a practical viewpoint, milk lipids are very important as they confer distinctive nutritional, textural and organoleptic properties on dairy products, such as cream, butter, whole milk powder and cheese.

The composition and structure of bovine milk fat have been reviewed extensively. There are early reviews by Morrison (1970), Christie (1978, 1995), Jensen and Clark (1988), and Jensen and Newberg (1995); recent articles include a comprehensive review of recent research by Jensen (2002) and two book chapters by Vanhoutte and Huyghebaert (2003), and Zegarska (2003). Bovine milk lipids are similar to the milk lipids of other species as they are largely composed of triacylglycerols; however, there are also minor amounts of diacylglycerols, monoacylglycerols, free (unesterified) fatty acids, phospholipids and

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

**Table 1.1.** Main classes of lipids in milk<sup>a</sup>

Lipid class	Amount (% w/w)
Triacylglycerols	98.3
Diacylglycerols	0.3
Monoacylglycerols	0.03
Free fatty acids	0.1
Phospholipids	0.8
Sterols	0.3
Carotenoids	trace
Fat-soluble vitamins	trace
Flavour compounds	trace

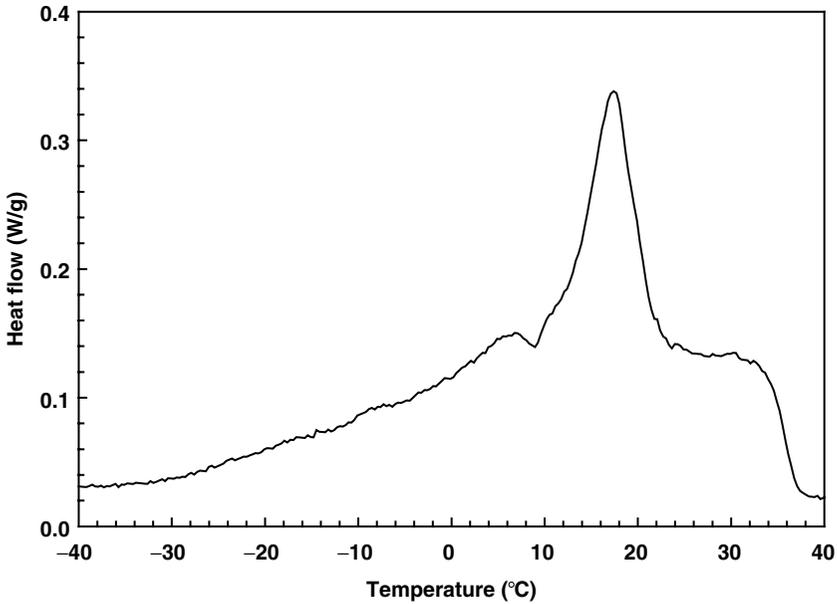
<sup>a</sup> Walstra and Jenness (1984)

sterols. Trace amounts of fat-soluble vitamins,  $\beta$ -carotene and fat-soluble flavouring compounds are also present in the bovine milk lipids (Table 1.1).

Because the triacylglycerols account for about 98% of the total fat, they have a major and direct effect on the properties of milk fat, for example hydrophobicity, density and melting characteristics. These triacylglycerols are a complex mixture, and vary considerably in molecular weight and degree of unsaturation. After milking, fresh milk contains only small amounts of diacylglycerols and monoacylglycerols and free fatty acids. The small proportion of diacylglycerols are largely *sn*-1,2 diacylglycerols and are, therefore, probably intermediates in the biosynthesis of triacylglycerols rather than the products of lipolysis (Lok, 1979). The profile of free fatty acids in freshly-drawn milk differs somewhat from the profile of the fatty acids esterified to the triacylglycerols (e.g., there appears to be very little free butanoic acid), also indicating that they are unlikely to be the result of lipase action (Walstra and Jenness, 1984).

Phospholipids account for only 0.8% of milk lipids. However, they play a major role in milk due to their amphiphilic properties. About 65% of them are found in the milk fat globule membrane (MFGM), whereas the rest remain in the aqueous phase. Phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin are the major phospholipids of milk, which together comprise about 90% of the total. Sterols are also a minor component, comprising about 0.3% of the fat; cholesterol, being the principal sterol, accounts for over 95% of the total sterols.

Milk fat is present in spherical droplets, which range from about 0.2 to 15.0  $\mu\text{m}$  in diameter, with the bulk of the fat being in globules 1.0 to 8.0  $\mu\text{m}$  diameter. The MFGM, which envelopes the fat globule, consists largely of proteins and lipids. The protein of the membrane has a complex composition and over 40 polypeptides have been identified. Xanthine oxidoreductase,



**Figure 1.1.** Melting profile of New Zealand milk fat, determined by differential scanning calorimetry (MacGibbon, 1988).

butyrophilin, PAS 6 and PAS 7 are found to be the major proteins. The lipids in the membrane are largely phospholipids and triacylglycerols. In contrast to the MFGM, the fat globule core almost exclusively consists of triacylglycerols (Keenan and Dylewski, 1995; see Chapter 4, Keenan and Mather).

The chemical properties of milk lipids can have a considerable influence on the melting characteristics of milk fat, which in turn can have a marked effect on the functional properties of a number of dairy products, such as cheese and butter (Chen *et al.*, 2004). Milk fat melts over a wide range, from about  $-35^{\circ}\text{C}$  to  $38^{\circ}\text{C}$  (Figure 1.1). There is a small broad peak centred at about  $7^{\circ}\text{C}$ , a major melting peak at about  $17^{\circ}\text{C}$ , and a plateau from  $22^{\circ}\text{C}$  to  $36^{\circ}\text{C}$ . It can be seen that a substantial proportion of milk fat melts between  $10^{\circ}\text{C}$  and  $20^{\circ}\text{C}$ . This broad melting range is directly attributable to the large number of different types of triacylglycerols present in the milk fat.

## 1.2. Fatty Acids

Bovine milk fat is regarded as one of the most complex naturally-occurring fats and oils, because of the large number of fatty acids with a variety of structures. Using a combination of chromatographic and spectroscopic

techniques, researchers have identified approximately 400 fatty acids in milk fat. A listing of the various types of fatty acids has been compiled by Jensen (2002). The vast majority of these acids are present in extremely small quantities (<0.01%). However, there are about 15 fatty acids that are present at or above 1.0% concentration. The quantities of these “major” fatty acids are determined relatively easily by capillary gas chromatography (GC) (IDF, 2002). Percentages for these fatty acids in milk fat are shown in Table 1.2. The typical values are for cows in mid-lactation, grazing on mature pasture. The range of values is for the dairying season in New Zealand, where cows graze on pasture throughout the year.

### 1.2.1. Origins of the Fatty Acids

The fatty acids of bovine milk fat arise from two sources: synthesis *de novo* in the mammary glands and the plasma lipids originating from the feed. The fatty acids from these two sources differ in their structure. The fatty acids that are synthesised *de novo* are short-chain and medium-chain length acids, from 4:0 to 14:0 and also some 16:0, while the C<sub>18</sub> fatty acids and some 16:0 arise from the plasma lipids. *De novo* fatty acid synthesis accounts for approximately 45% (w/w) of the total fatty acids in milk fat, while lipids of dietary origin account for the rest (Moore and Christie, 1979).

The *de novo* synthesis of fatty acids in the mammary gland utilizes mainly acetate and some  $\beta$ -hydroxybutyrate. These precursors arise from the microbial fermentation of cellulose and related materials in the rumen. Once in the mammary gland, acetate is activated to acetyl-CoA. The mechanism of fatty acid synthesis essentially involves the carboxylation of acetyl-CoA to malonyl-CoA, which is then used in a step-wise chain elongation process. This leads to a series of short-chain and medium-chain length fatty acids, which differ by two CH<sub>2</sub> groups (e.g., 4:0, 6:0, 8:0, etc.) (Hawke and Taylor, 1995). These are straight-chain, even-numbered carbon fatty acids. However, if a precursor such as propionate, valerate or isobutyrate, rather than acetate, is used, branched-chain or odd-numbered carbon fatty acids are synthesised (Jenkins, 1993; see Chapter 2).

Other fatty acids originate mainly from the source of diet, although these include fatty acids that can also be released from adipose tissues. Dietary lipids consist largely of glycolipids, phospholipids and triacylglycerols, and the major fatty acids are linoleic acid (9*c*, 12*c*-18:2) and linolenic acid (9*c*, 12*c*, 15*c*-18:3). In the rumen, these lipids are hydrolyzed initially to produce non-esterified fatty acids, which are then subjected to extensive biohydrogenation by micro-organisms (Jenkins, 1993). The biohydrogenation sequence for linoleic acid begins with an isomerisation step, which produces conjugated linoleic acid (9*c*, 11*t*-18:2). This is followed by a

reduction reaction to give vaccenic acid (11*t*-18:1), and then a further reduction to 18:0. Biohydrogenation of linolenic acid follows a similar pathway (Bauman *et al.*, 1999; see Chapter 3, Bauman & Lock). The mix of fatty acids that results from biohydrogenation is esterified to triacylglycerols, which then circulate in the bloodstream within chylomicrons. These triacylglycerols are taken up by the mammary gland and cleaved to give non-esterified fatty acids. The mammary gland contains a desaturase system, which converts substantial quantities of 18:0 to oleic acid (9*c*-18:1).

The net result of these processes is that the fatty acids in the mammary gland, which originate from the dietary lipids, consist of substantial quantities of 16:0, 18:0 and oleic acid, small amounts of linoleic and linolenic acids, and limited quantities of other monoenoic and dienoic fatty acids such as 11*t*-18:1 and 9*c*, 11*t*-18:2.

### 1.2.2. Saturated Fatty Acids

The saturated fatty acids that are present in significant quantities in milk fat are molecules with un-branched hydrocarbon chains, which vary in length from 4 to 18 carbon atoms. These fatty acids account for approximately 70 to 75% of the total fatty acids. The most important saturated fatty acid from a quantitative viewpoint is 16:0, which accounts for about 25 to 30% of the total, while two other fatty acids, 14:0 and 18:0 have values in the region 10 to 13% (Table 1.2). The amounts of the short-chain fatty acids, 4:0 and 6:0, are reasonably high when their proportions are expressed as molar percentages (approximately 10 and 5%, respectively—Table 1.2); appreciable amounts of medium-chain length fatty acids (C<sub>8</sub> to C<sub>12</sub>) are also present.

Short-chain and medium-chain fatty acids in milk fat have certain interesting characteristics, which may explain some of the reasons for their presence. Unlike long-chain fatty acids, short-chain and medium-chain fatty acids are absorbed as non-esterified fatty acids into the portal bloodstream and are metabolised rapidly in the liver (Noble, 1978). Hence, they are able to make a direct and rapid contribution to the energy metabolism of the new-born calf. Furthermore, short-chain fatty acids and, to a lesser extent, medium-chain fatty acids lower the melting point of triacylglycerols and, thus, their presence helps keep milk fat liquid at physiological temperatures. This helps in compensating the relatively low concentration of low melting point, unsaturated fatty acids in milk fat.

### 1.2.3. *Cis*-unsaturated Fatty Acids

The *cis*-monoenoic acid content of bovine milk fat is about 18 to 24% (Table 1.2). Oleic acid (9*c*-18:1) is the principal *cis*-monounsaturated fatty acid, accounting for around 15–21% of the total. There is

**Table 1.2.** Major fatty acids in bovine milk fat

	Common Name	Composition		
		Typical <sup>a</sup>		Range <sup>b,c</sup>
		%(w/w)	mol %	%(w/w)
4:0	Butyric	3.9	10.1	3.1–4.4
6:0	Caproic	2.5	4.9	1.8–2.7
8:0	Caprylic	1.5	2.4	1.0–1.7
10:0	Capric	3.2	4.3	2.2–3.8
12:0	Lauric	3.6	4.1	2.6–4.2
14:0	Myristic	11.1	11.1	9.1–11.9
14:1	Myristoleic	0.8	0.8	0.5–1.1
15:0	–	1.2	1.1	0.9–1.4
16:0	Palmitic	27.9	24.9	23.6–31.4
16:1	Palmitoleic	1.5	1.4	1.4–2.0
18:0	Stearic	12.2	9.8	10.4–14.6
18:1 <i>cis</i>	Oleic	17.2	13.9	14.9–22.0
18:1 <i>trans</i>		3.9	3.2	
18:2	Linoleic	1.4	1.1	1.2–1.7
18:2 conj	Conjugated Linoleic acid	1.1	0.9	0.8–1.5
18:3	$\alpha$ Linolenic	1.0	0.8	0.9–1.2
	Minor acids	6.0	5.1	4.8–7.5

<sup>a</sup> Creamer and MacGibbon (1996).

<sup>b</sup> MacGibbon (unpublished).

<sup>c</sup> Range of values for dairying season.

about 0.5% of 11*c*-18:1, while the proportions of other *cis*-18:1 isomers are small. There are also relatively small but significant contributions from other *cis*- monounsaturated acids, namely 14:1 (about 1.0%) and 16:1 (about 1.5%).

*Cis*-polyenoic acids are present at low concentrations in milk fat, because of the biohydrogenation reactions that take place in the rumen. These acids are comprised almost exclusively of linoleic acid (9*c*, 12*c*-18:2), about 1.2 to 1.7% and  $\alpha$ -linolenic acid (9*c*, 12*c*, 15*c*-18:3), about 0.9 to 1.2% (Table 1.2). These two fatty acids are essential fatty acids; they cannot be synthesised within the body and must be supplied by the diet. In recent times, the usage of the term “essential” has been extended to include derivatives of these fatty acids, which are not synthesised in significant quantities (e.g., eicosapentaenoic acid, 20:5 and docosahexaenoic acid, 22:6). The proportion of  $\alpha$ -linolenic acid appears to be affected by the cow’s diet; the concentration is higher in milk from pasture-fed cows than in milk from barn-fed cows (Hebeisen *et al.*, 1993; Wolff *et al.*, 1995). In the case of linoleic

**Table 1.3.** Concentration of *trans*-octadecenoic acids in bovine milk fat<sup>a</sup>

	<i>Trans</i> -octadecenoic acid isomers										Total
	Composition (%(w/w) of total fatty acids)										
	Δ4	Δ5	Δ6-8	Δ9	Δ10	Δ11	Δ12	Δ13/14	Δ15	Δ16	
Mean value	0.05	0.05	0.17	0.24	0.17	1.75	0.21	0.48	0.28	0.34	3.74
Max value	0.13	0.12	0.30	0.31	0.26	4.00	0.31	0.73	0.47	0.51	6.34
Min value	0.02	0.02	0.03	0.16	0.00	0.52	0.11	0.25	0.10	0.16	1.91

<sup>a</sup> Precht and Molkentin (1996); 100 samples were analyzed.

acid, the picture is less clear with differing trends being reported by the two research groups.

#### 1.2.4. *Trans*-unsaturated Fatty Acids

The presence of C<sub>18</sub> *trans*-fatty acids in milk fat is the result of incomplete biohydrogenation of the unsaturated dietary lipids in the rumen. These fatty acids have attracted attention because of their adverse nutritional affects. Clinical trials have shown that *trans*-octadecenoic acids, relative to the *cis* isomer, can increase the LDL-cholesterol and decrease the HDL-cholesterol, thus, producing an unfavourable affect on the LDL:HDL ratio (Mensink and Katan, 1993).

The quantitative determination of individual isomers of *trans*-18:1 fatty acids in milk fat is not straightforward. It involves a multi-stage analytical procedure (i.e., transesterification of milk fat, argentation TLC of the fatty acid esters, to separate the *cis*-isomers and *trans*-isomers, followed by capillary GC). This method gives an almost complete separation of the 13 individual *trans*-18:1 isomers, from Δ4 to Δ16 (Precht and Molkentin, 1996).

Vaccenic acid (11*t*-18:1) is the most important *trans* isomer with values ranging from about 30 to 60% of the total *trans*-18:1 (Table 1.3). The concentration of *trans*-18:1 varies considerably from about 2.0 to 6.0%, with mean values for milk fats from several European countries in the range 3.3 to 4.4% (Precht and Molkentin, 2000). The higher values are for milk fat samples that were obtained from cows fed on summer pasture, whereas the lower values were associated with the feeding of concentrates and silage to cows in the winter. The feeding of fresh grass to cows appears to reduce the efficiency of the biohydrogenation reactions in the rumen, which leads to higher amounts of *trans* fatty acids.

Although the level of *trans*-18:1 in milk fat is significant, it is found well below the level that is present in some margarines. In a study of Canadian margarines, Ratnayake and Pelletier (1992) found that the concentration of *trans*-18:1 ranged from 7 to 35% for soft margarines, and from 25 to 42% for hard margarines. These *trans* acids are the result of partial hydrogenation of vegetable oils to produce a hard stock for use in semi-solid fat products, such as margarine and confectionery.

Precht and Molkenntin (1997) have identified and quantified a number of *trans*-octadecadienoic acids in milk fat, containing one or two *trans*-double bonds (Table 1.4). Most of these acids are present in small amounts, with only 11*t*, 15*c*-18:2 (0.33%) and 9*c*, 11*t*-18:2 (0.85%) having average concentrations above 0.30%. These *trans*-octadecadienoic acids show considerable variation; for instance, 9*c*, 11*t* 18:2 ranges from 0.25 to 1.95%. Similar to *trans*-octadecenoic acids, pasture feeding produces higher levels of *trans*-octadecadienoic acids compared to the feeding of mixed rations.

9*c*, 11*t*-18:2 is the principal isomer of the conjugated linoleic acids (CLA) in bovine milk fat, accounting for about 80–90% of the total (Parodi, 1977). The term CLA refers to a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds (e.g., 10*t*, 12*c*-18:2, 10*t*, 12*t*-18:2). The CLA content of milk fat is derived from two related sources. First, 9*c*, 11*t*-18:2 is an intermediate product of the biohydrogenation of fatty acids in the rumen. In addition, 11*t*-18:1 can be converted to 9*c*, 11*t*-18:2 in the mammary gland by the enzyme stearoyl-CoA desaturase (SCD), which normally catalyzes the conversion of 18:0 to 9*c*-18:1 (see chapter 3).

The presence of 9*c*, 11*t*-18:2 in bovine milk fat has been known for several years (Parodi, 1977). However, it was only after Ha *et al.*

**Table 1.4.** Concentration of *trans*-octadecadienoic acids in bovine milk fat<sup>a</sup>

<i>Trans</i> -18:2 isomers	n	Composition (% w/w, of total fatty acids)		
		Mean	Max	Min
9 <i>c</i> , 11 <i>t</i> -18:2	100	0.85	1.95	0.25
9 <i>c</i> , 12 <i>t</i> -18:2	100	0.10	0.16	0.05
9 <i>t</i> , 12 <i>c</i> -18:2	100	0.07	0.48	0.02
9 <i>c</i> , 13 <i>t</i> -18:2 } 8 <i>t</i> , 12 <i>c</i> -18:2 }	11	0.11	0.16	0.07
11 <i>t</i> , 15 <i>c</i> -18:2	100	0.33	0.68	0.04
9 <i>t</i> , 12 <i>t</i> -18:2	11	0.09	0.12	0.06
<i>t</i> , <i>t</i> ,-NMID <sup>b</sup>	11	0.19	0.38	0.10

<sup>a</sup> Precht and Molkenntin (1997).

<sup>b</sup> NMID – Non-methylene interrupted diene.

(1987) identified 9*c*, 11*t*-18:2 as an anti-carcinogenic substance in minced beef that there was an upsurge in scientific investigations. In subsequent work, the health implications of CLA have widened to include inhibition of carcinogenesis, atherosclerosis, diabetes and weight loss induced by immune stimulation; and increase in the percentage of lean body mass (Parodi, 1999). These studies have shown that CLA can be regarded as unique, because it appears to provide numerous positive health effects unlike other *trans* acids. This research is discussed in Chapter 3 (Bauman and Lock).

An important structural difference between *cis*-unsaturated and *trans*-unsaturated fatty acids is that the *cis* configuration of the double bond puts a significant “kink” in the hydrocarbon chain, whereas the *trans* configuration causes only a slight distortion. This difference has a major impact on the way in which triacylglycerols pack in crystal lattices when they solidify. Triacylglycerols containing *cis*-unsaturated fatty acids have a lower packing density than triacylglycerols containing either *trans*-unsaturated or saturated fatty acids, and as a result have lower melting points. Thus, the relative levels of *cis*-unsaturated and *trans*-unsaturated fatty acids can exert a considerable influence on the melting characteristics of milk fat.

### 1.2.5. Minor Fatty Acids

As noted earlier, there are approximately 400 minor fatty acids in bovine milk fat; about 40 are present at levels >0.01% (Table 1.5), while the remainder exist in trace amounts. Most of these fatty acids are of little practical importance and, hence, their nature and structure is of academic interest only.

Among the minor saturated fatty acids are branched-chain and odd-numbered carbon fatty acids with a range of chain length from C<sub>3</sub> to C<sub>27</sub>. Examples of odd-numbered fatty acids are 13:0 (0.19%), 17:0 (0.6%) and 19:0 (0.15%) (Table 1.5). The monomethyl branched-chain fatty acids are quite significant, accounting for about 2.5% of the total fatty acids. Examples are the C<sub>15</sub> branched-chain fatty acids, 13-methyl tetradecanoic acid (the *iso* configuration) and 12-methyl tetradecanoic acid (the *anteiso* configuration), which together make up about 0.8% of milk fat (Table 1.5).

There are about 200 minor monoenoic, dienoic and polyenoic fatty acids in milk fat ranging in chain length from C<sub>10</sub> to C<sub>24</sub>, and consisting of both positional and *cis/trans* isomers. A number have considerable nutritional significance; for example, eicosapentaenoic acid (20:5, 0.09%) and docosahexaenoic acid (22:6, 0.01%) are present in the metabolic pathway of the n-3 fatty acids, while arachidonic acid (20:4, 0.14%) is part of the n-6 pathway.

Jensen (2002) has reported that milk fat contains about 60 hydroxy fatty acids. The C<sub>4</sub>-hydroxy and C<sub>5</sub>-hydroxy acids are of interest as they

**Table 1.5.** Minor fatty acids in bovine milk fat<sup>a</sup>

Composition (% w/w, of total fatty acids)							
Saturated				Unsaturated			
Straight-chain		Branched-chain <sup>b</sup>		Monounsaturated		Polyunsaturated	
FA	%(w/w)	FA	%(w/w)	FA	%(w/w)	FA	%(w/w)
11:0	0.20	13:0i	0.03	10:1	0.15	20:2	0.07
13:0	0.19	14:0a	0.02	12:1	0.06	20:3	0.10
17:0	0.60	15:0i	0.40	13:1	0.03	20:4	0.14
19:0	0.15	15:0a	0.44	17:1	0.36	20:5	0.09
20:0	0.35	16:0i	0.40	19:1	0.16	22:2	0.04
21:0	0.04	17:0i	0.50	20:1	0.32	22:3	0.07
22:0	0.20	17:0a	0.52	21:1	0.04	22:4	0.03
23:0	0.12	18:0i	0.16	22:1	0.06	22:5	0.04
24:0	0.14	19:0i	0.10			22:6	0.01
25:0	0.03						
26:0	0.06						

<sup>a</sup> Iverson and Sheppard (1986). Minor fatty acids present at levels  $\geq 0.01\%$ .

<sup>b</sup> i = iso, a = anteiso

transform to the respective 4-carbon ( $\gamma$ ) and 5-carbon ( $\delta$ ) lactones, which are major contributors to the overall flavour of the milk fat. Approximately 60 keto (oxo) acids have been isolated and identified in milk fat (Weihrach, 1974; Brechany and Christie, 1992). When milk fat is heated,  $\beta$ -keto acids are decarboxylated to form methyl ketones, which contribute to cooked butter flavours.

### 1.2.6. Variations In Fatty Acid Composition

The fatty acid composition of milk fat is not stable and is influenced by a number of factors. These include, breed of cow, stage of lactation and type, and quality and quantity of feed (Grummer, 1991; Beaulieu and Palmquist, 1995; Hawke and Taylor, 1995; Auldust *et al.*, 1998; Zegarska *et al.*, 2001). These issues are discussed in detail in Chapter 2 (Palmquist).

In most countries, there exists a regularly recurring seasonal pattern of fatty acid variation in milk fat, which is caused largely by changes to the cow's diet. This seasonal variation can have an impact on the properties of high-fat dairy products, e.g., the hardness of butter (MacGibbon and McLennan, 1987). The seasonal variation for French milk fat is presented in Table 1.6 (Wolff *et al.*, 1995). It can be seen that 16:0 has a markedly lower value in spring and summer than in winter. The C<sub>6</sub> to C<sub>14</sub> fatty acids together show a similar trend, although the magnitude of the change is much smaller. In

**Table 1.6.** Fatty acid composition of French butters collected at different periods of the year<sup>a</sup>

Fatty Acid	Composition (% w/w)				
	January	March	May–June	July–August	Oct–Nov
4:0	4.0	4.4	3.8	4.2	4.3
6:0	2.5	2.7	2.4	2.5	2.6
8:0	1.5	1.6	1.4	1.5	1.5
10:0	3.5	3.5	3.1	3.1	3.3
12:0	4.0	4.0	3.6	3.5	3.8
14:0	12.1	11.9	11.0	11.2	11.3
14:1	1.1	1.0	0.9	1.1	0.9
15:0	1.2	1.2	1.2	1.2	1.1
16:0	33.3	32.5	27.1	28.3	29.3
16:1	1.5	1.6	1.4	1.5	1.7
18:0	9.0	9.0	11.0	10.5	9.6
18:1 <i>cis</i>	16.8	17.3	19.7	19.2	19.4
18:1 <i>trans</i>	2.4	2.4	4.3	3.7	3.2
18:2	1.4	1.5	1.2	1.3	1.4
18:2 conj	0.4	0.5	0.7	0.7	0.5
18:3	0.3	0.3	0.6	0.5	0.5
Minor	5.0	4.6	6.6	6.0	5.6

<sup>a</sup> Wolff *et al.* (1995).

contrast, 18:0 and 18:1 (*cis* and *trans*) show a reverse trend with lower levels in winter. Generally, higher values of 16:0 tend to be associated with higher levels of total lipids and greater hardness of the fat. These variations are attributed to a change from winter feed of hay and concentrate to a diet of fresh grass in spring. The lipids in fresh grass contain high levels of 18:2 and 18:3 which, as a result of biohydrogenation and desaturation reactions in the cow, lead to increased levels of 18:0 and 18:1 in milk fat.

Similar seasonal trends in fatty acid composition have been found in other countries where the pattern of dairy husbandry practices is similar (Hughebaert and Hendrickx, 1971; Muuse *et al.*, 1986; Lindmark-Mansson *et al.*, 2003).

### 1.3. Triacylglycerols

Bovine milk fat contains various triacylglycerols, which vary considerably in molecular weight and degree of unsaturation. This complexity is the direct result of the large number and wide variety of fatty acids which make up the triacylglycerols.

**Table 1.7.** General composition of triacylglycerols of bovine milk fat

Carbon Number	Triacylglycerol Composition % (w/w)			
	German milk fat <sup>a</sup>		New Zealand milk fat <sup>b</sup>	
	Average	Range <sup>c</sup>	Typical	Range <sup>d</sup>
C26	0.2	0.2–0.3	–	–
C28	0.6	0.5–0.8	0.6	0.4–0.8
C30	1.2	1.0–1.9	1.2	0.8–1.4
C32	2.6	2.1–3.2	2.5	1.8–2.9
C34	6.0	4.8–6.9	5.8	4.4–6.4
C36	10.9	9.2–12.4	11.0	9.1–11.8
C38	12.8	12.1–13.6	13.3	11.8–14.6
C40	10.1	9.5–11.2	10.7	9.7–12.1
C42	7.1	6.2–7.9	7.4	6.5–7.9
C44	6.7	5.4–7.8	6.7	5.6–7.3
C46	7.4	6.3–8.3	7.2	5.6–7.8
C48	9.1	8.0–10.7	8.6	6.9–9.9
C50	10.9	9.7–12.0	10.6	9.7–12.8
C52	9.5	7.2–12.3	9.4	7.7–12.6
C54	4.6	2.7–7.8	4.7	3.7–7.0
C56	–	–	0.4	0.4–0.6

<sup>a</sup> Precht and Frede (1994)

<sup>b</sup> MacGibbon (unpublished)

<sup>c</sup> Range of values for different regions.

<sup>d</sup> Range of values over a dairying season.

As noted earlier, there are some 400 fatty acids in milk fat, which means that theoretically milk fat could contain many thousand triacylglycerols. Even if one considers only the 15 or so fatty acids that are present at concentrations above 1% (Table 1.2), and ignores the placement of these fatty acids at specific positions on the triacylglycerol molecule, there are still 680 compositionally different triacylglycerols.

The general composition of triacylglycerols can readily be determined by capillary GC. Typical triacylglycerol compositions of milk fats from Germany and New Zealand are presented in Table 1.7. The triacylglycerols show a wide molecular weight range (from acyl carbon 26 to 56), which arises from the large differences in chain length of the constituent fatty acids (from C<sub>4</sub> to C<sub>18</sub>). The triacylglycerol composition is dominated by triacylglycerols with 36 to 40 acyl carbons (about 35% of the total) and 46 to 52 acyl carbons (about 36% of the total). The range of values for the different carbon numbers is considerable, indicating that there is significant variation in triacylglycerol composition both throughout the dairying season and between different dairying regions. Interestingly, data for the two countries are remarkably similar.

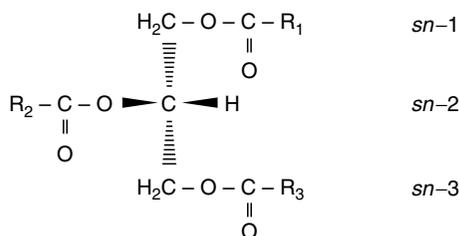
It should be noted that simple capillary GC, while convenient, just separates triacylglycerols into groups of similar molecular weight, and does not provide information on individual triacylglycerols – carbon number 38, for example, will consist of several different triacylglycerols (e.g., 4:0, 16:0, 18:0; 4:0, 16:0, 18:1; 6:0, 14:0, 18:1, etc.).

### 1.3.1. Structure of Triacylglycerols

Triacylglycerols are synthesised in the mammary gland by enzymic mechanisms that exert some selectivity over the esterification of different fatty acids at each position of the *sn*-glycerol moiety (Moore and Christie, 1979). A triacylglycerol molecule showing the three *sn*-positions is shown in Figure 1.2.

Stereospecific analytical procedures have been developed that have enabled the determination of the positional distributions of fatty acids on the triacylglycerols. The results obtained using these procedures show that there is a highly selective stereospecific distribution of fatty acids in the triacylglycerols of bovine milk fat (Table 1.8). For cows fed a normal diet, the fatty acids 4:0 and 6:0 are esterified almost entirely at position *sn*-3. In contrast, 12:0 and 14:0 are esterified preferentially at position *sn*-2, while 16:0 is incorporated preferentially at positions *sn*-1 and *sn*-2. 18:0 is esterified preferentially at position *sn*-1, and 18:1 shows a preference for positions *sn*-1 and *sn*-3. This overall pattern of fatty acid distribution does not change significantly either throughout the dairying season or between countries (Pitas *et al.*, 1967; Taylor and Hawke, 1975b; Parodi, 1979; Christie and Clapperton, 1982).

Stereospecific analysis of milk fat fractions containing triacylglycerols of different molecular weight have shown that, for fatty acids of chain length C<sub>4</sub> to C<sub>16</sub>, the general pattern of fatty acid distribution in normal milk fat is similar to the pattern of distribution in the triacylglycerol fractions of different



**Figure 1.2.** Fischer projection diagram of a triacylglycerol showing the stereospecific numbering (*sn*-) convention.

**Table 1.8.** Positional distribution of fatty acids in the triacylglycerols of bovine milk fat<sup>a</sup>

Fatty Acid	Fatty Acid Composition (mol %)		
	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3
4:0	–	0.4	30.6
6:0	–	0.7	13.8
8:0	0.3	3.5	4.2
10:0	1.4	8.1	7.5
12:0	3.5	9.5	4.5
14:0	13.1	25.6	6.9
16:0	43.8	38.9	9.3
18:0	17.6	4.6	6.0
18:1	19.7	8.4	17.1

<sup>a</sup> Calculated from the data of Parodi (1979).

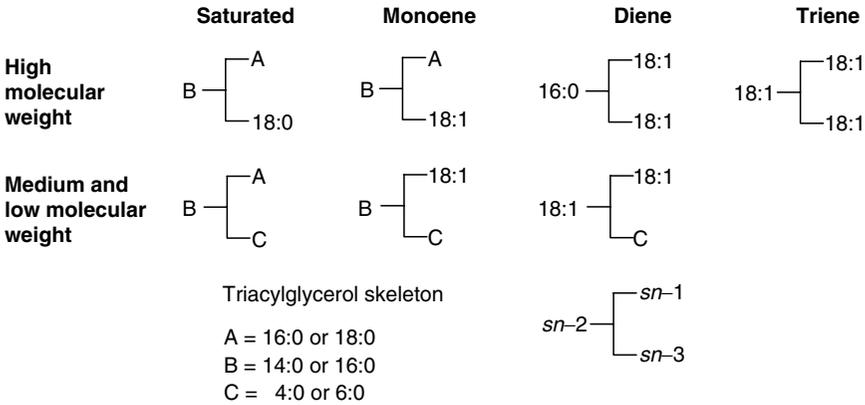
molecular weight. However, the pattern of distribution of 18:0 and 18:1 varies according to the molecular weight of the triacylglycerols; these fatty acids tend to be esterified preferentially at positions *sn*-1 and *sn*-3 in triacylglycerols of high molecular weight and concentrated at position *sn*-1 in triacylglycerols of medium- and low-molecular weight (Parodi, 1982).

### 1.3.2. Composition of Triacylglycerols

As noted earlier, milk fat contains a very complex mixture of triacylglycerols. This complexity has made the identification and characterization of individual triacylglycerols extremely difficult. Moreover, the fact that no two batches of milk fat have exactly the same composition adds to the difficulty. As a result, the majority of the earlier studies were aimed at elucidating the general types of triacylglycerols present rather than obtaining quantitative data about individual triacylglycerols.

In a series of investigations, milk fat was fractionated into different triacylglycerol classes on the basis of molecular weight and degree of unsaturation, using a combination of chromatographic methods, namely normal and argentation TLC, and GC. This approach, in combination with stereospecific analysis, provided detailed information on the different classes of triacylglycerols present in milk fat (Breckenridge and Kuksis, 1968, 1969; Taylor and Hawke, 1975a; Parodi, 1980).

The high molecular weight fractions of differing degrees of unsaturation were found to consist largely of triacylglycerols containing combinations of four long-chain fatty acids, namely 14:0, 16:0, 18:0 and 18:1. The most likely placement of these fatty acids at the different positions on the triacylglycerol



**Figure 1.3.** Probable composition of the major triacylglycerols of milk fat.

molecules is shown in Figure 1.3. On the other hand, the medium- and low-molecular weight fractions were comprised mainly of triacylglycerols with combinations of these four long-chain fatty acids at positions *sn*-1 and *sn*-2 and a short-chain fatty acid (either 4:0 or 6:0) esterified at position *sn*-3.

The saturated and monoene triacylglycerol classes were dominant and each comprised about 35 to 40% of the total milk fat, while the approximate proportions of the high-, medium- and low-molecular weight fractions were 40, 20 and 40%, respectively.

More recently, the use of more sophisticated chromatographic techniques, particularly HPLC and capillary GC, has led to the identification and quantification of individual, compositionally-different triacylglycerols. In one painstaking study, Gresti *et al.* (1993) separated milk fat by reversed-phase HPLC into 47 fractions. Each fraction was then analysed for triacylglycerol and fatty acid composition by capillary GC. The data obtained were used to calculate the proportions of some 220 individual molecular species of triacylglycerols, accounting for 80% of the total triacylglycerols in the sample. The quantitatively important triacylglycerols, each present at >0.5%, are shown in Table 1.9. This list of 40 major triacylglycerols makes up about 55% of the total milk fat. An interesting aspect of the data is that some triacylglycerols are present in high proportions, for example 4:0, 16:0, 18:1 (4.2%); 4:0, 16:0, 16:0 (3.2%); 4:0, 14:0, 16:0 (3.1%); 14:0, 16:0, 18:1 (2.8%); 4:0, 16:0, 18:0 (2.5%); and 16:0, 18:1, 18:1 (2.5%). Although, this was a long and exhaustive study, it did not definitively identify the constituent triacylglycerols of milk fat as the placement of the fatty acids at the different *sn*-positions on the triacylglycerol molecules was not determined.

**Table 1.9.** Proportions (mol %) of the major triacylglycerols in a sample of French milk fat<sup>a,b,c</sup>

Carbon Number	Saturated Triacylglycerols		Monoene Triacylglycerols			Diene and Triene Triacylglycerols	
C30	4:0, 10:0, 16:0	0.6%					
C32	4:0, 12:0, 16:0	0.8%					
C34	4:0, 14:0, 16:0	3.1%					
C36	4:0, 14:0, 18:0	1.3%	4:0, 14:0, 18:1	1.8%			
	4:0, 16:0, 16:0	3.2%					
	6:0, 14:0, 16:0	1.4%					
C38	4:0, 16:0, 18:0	2.5%	4:0, 16:0, 18:1	4.2%			
	6:0, 16:0, 16:0	1.5%	6:0, 14:0, 18:1	0.9%			
	6:0, 14:0, 18:0	0.6%					
C40	6:0, 16:0, 18:0	1.1%	4:0, 18:0, 18:1	1.6%	4:0, 18:1, 18:1	1.5%	
	8:0, 16:0, 16:0	0.7%	6:0, 16:0, 18:1	2.0%			
	10:0, 14:0, 16:0	0.7%					
C42	10:0, 16:0, 16:0	1.0%	10:0, 14:0, 18:1	0.6%	6:0, 18:1, 18:1	0.6%	
	12:0, 14:0, 16:0	0.6%					
C44	14:0, 14:0, 16:0	0.6%	10:0, 16:0, 18:1	1.6%			
C46	14:0, 16:0, 16:0	0.9%	12:0, 16:0, 18:1	1.2%	10:0, 18:1, 18:1	0.7%	
			14:0, 14:0, 18:1	0.6%			
C48	14:0, 16:0, 18:0	0.7%	14:0, 16:0, 18:1	2.8%	12:0, 18:1, 18:1	0.6%	
C50			14:0, 18:0, 18:1	1.4%	14:0, 18:1, 18:1	1.2%	
C52			16:0, 16:0, 18:1	2.3%			
			16:0, 18:0, 18:1	2.2%	16:0, 18:1, 18:1	2.5%	
C54					16:0, 18:1, 18:2	0.6%	
			18:0, 18:0, 18:1	0.8%	18:0, 18:1, 18:1	1.2%	
					18:1, 18:1, 18:1	1.0%	

<sup>a</sup> Gresti *et al.* (1993).<sup>b</sup> Triacylglycerols at concentrations >0.5%.<sup>c</sup> Position of fatty acid on triacylglycerol molecule not determined.

A number of recent investigations have shown that mass spectrometry (MS) is a rapid and effective method for the identification of triacylglycerol species of milk fat that are compositionally different (Myher *et al.*, 1988, 1993; Laakso and Kallio, 1993; Spanos *et al.*, 1995; Laakso and Manninen, 1997; Mottram and Evershed, 2001; Kalo *et al.*, 2004). In fact, a range of mass spectral techniques, such as electron ionization, fast atom bombardment, chemical ionization, atmospheric pressure chemical ionization and electrospray MS, have been used to study triacylglycerols. The later three are “soft” ionizing techniques, which retain substantial amounts of the molecular ion, rather than fragmenting the molecule into a number of parts. These methods have allowed the determination of

fatty acids that contribute to the triacylglycerols, especially with the advent of MS/MS where the further fragmentation of a particular molecular ion can be effected, displaying fragments derived solely from a species or group (Kalo *et al.*, 2004).

In these mass spectral studies there are difficulties associated with the accurate quantification of the different types of triacylglycerols. First, MS shows a difference in sensitivity depending on the degree of unsaturation of triacylglycerols; fully saturated triacylglycerols tend to show a lower molecular ion response than unsaturated triacylglycerols (Byrdwell, 2001). Second, the studies using tandem MS/MS, in which diacylglycerol ions and fatty acid ions are formed from triacylglycerol ions, showed that the diacylglycerol ions were not representative of the expected random distribution of diacylglycerols, but rather contained more of the fatty acids at the *sn*-2 position. In other words, the release of fatty acids from the *sn*-2 position was less than the release from the *sn*-1 and *sn*-3 positions (Currie and Kallio, 1993).

Despite these concerns, several researchers have used MS to identify many of the constituent triacylglycerols of milk fat. These studies invariably begin with extensive fractionation of the triacylglycerols prior to mass spectral analysis, to ensure that the number of triacylglycerol species contributing to a particular fraction are as small as possible. In one of the earliest investigations, Myher *et al.* (1988) studied a milk fat fraction which was composed largely of low molecular weight triacylglycerols. After an initial separation using argentation TLC, which separated the triacylglycerols according to their degree of unsaturation, mass spectral analysis was used to identify more than 100 triacylglycerols.

In a further comprehensive investigation by Spanos *et al.* (1995), milk fat was fractionated initially by HPLC into 58 triacylglycerol fractions. These fractions were characterised using desorption chemical ionisation MS, followed by MS/MS if the peak contained a mixture of triacylglycerols. The fatty acids contributing to the triacylglycerols in each peak were determined and could be related to the acyl carbon number of the triacylglycerols. Thus, the composition of over 180 triacylglycerols were determined. Comparison of these results with the data of Gresti *et al.* (1993) showed that the triacylglycerols identified were similar. For example, for the C38 triacylglycerols, Gresti *et al.* (1993) found the following quantitatively-important triacylglycerols:- 4:0, 16:0, 18:1 (4.2%); 4:0, 16:0, 18:0 (2.5%); 6:0, 16:0, 16:0 (1.5%); 6:0, 14:0, 18:1 (0.9%); and 6:0, 14:0, 18:0 (0.6%). Although, Spanos *et al.* (1995) did not quantify the triacylglycerol species, only noting those that were present in greater amounts, they did identify the same group of C38 triacylglycerols as being present in significant quantities, with the exception of 4:0, 16:0, 18:0.

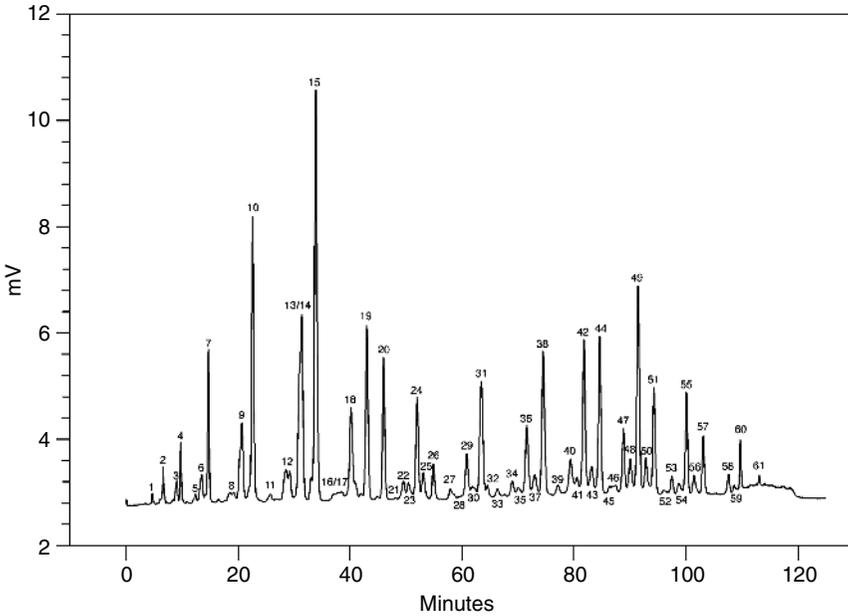
Mottram and Evershed (2001) undertook a similar study in which they fractionated milk fat by two different methods, silica TLC and gel

permeation chromatography. Each set of fractions was analyzed subsequently by an HPLC-MS system fitted with an atmospheric pressure chemical ionization source. Some fractions were also analyzed by GC-MS. This comprehensive analysis led to the identification of some 120 triacylglycerols.

Recently, Kalo *et al.* (2004) used normal-phase HPLC in combination with positive ion tandem MS to obtain quantitative information about the regioisomers of synthetic triacylglycerol mixtures and milk fat fractions containing low molecular weight triacylglycerols. In agreement with a previous study (Currie and Kallio, 1993), they found that the diacylglycerol fragment ions, produced by mass spectral analysis from standard triacylglycerol mixtures, contained greater amounts of fatty acids at the *sn*-2 position than predicted. Furthermore, the ratio of fatty acids at the *sn*-2 position, relative to the fatty acids at the *sn*-1 and *sn*-3 positions, varied according to the types of fatty acids attached. From the information gained about these diacylglycerols, the regioisomers of the synthetic triacylglycerol mixtures could be identified. In a similar manner, the regioisomers of the triacylglycerols in the milk fat fractions were studied, although the fatty acids at the *sn*-1 and *sn*-3 positions could not be differentiated.

Although these later detailed studies have not as yet yielded a method which definitively identifies and quantifies the constituent triacylglycerols of milk fat, the improvement in HPLC and mass spectral analyses have enabled researchers to develop routine methods that provide detailed information about milk fat triacylglycerols. One example is the method developed by Robinson and MacGibbon (1998), in which milk fat triacylglycerols were separated into 61 distinct peaks by reversed-phase HPLC (Figure 1.4). The triacylglycerols present in each peak were identified through initial fractionation by argentation TLC, followed by HPLC and MS. This HPLC method can be used as a single-injection, routine method, and appears to be sensitive enough to monitor relatively small changes in peak areas and, hence, minor changes in the amounts of small groups of triacylglycerols.

With the upsurge of interest in CLA, the distribution of CLA in milk fat triacylglycerols has also become a matter of considerable interest. The distribution of CLA has been determined by a reversed-phase HPLC system, in which the eluting peaks were simultaneously detected by both evaporative light scattering detection (ELSD) and UV absorption at 233 nm (Robinson and MacGibbon, 2000). The UV absorption data clearly show which peaks contain esterified CLA (the molar extinction coefficient for CLA at 233 nm is  $23,360 \text{ L mol}^{-1} \text{ cm}^{-1}$ ). The combined data from the two detection systems show that CLA is found in many different types of triacylglycerols, which differ in both molecular weight and degree of unsaturation.

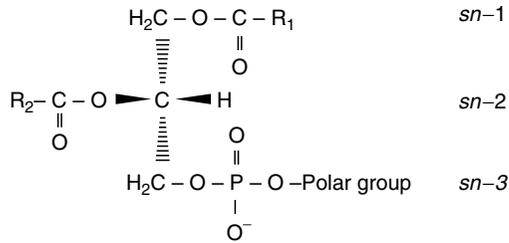


**Figure 1.4.** Reversed-phase HPLC chromatogram of milk fat triacylglycerols (from Robinson and MacGibbon, 1998).

### 1.4. Polar Lipids

The concentration of phospholipids in the milk fat ranges from 0.5 to 1.0% of the total (Patton and Jensen, 1976; Table 1.1). About 60 to 65% of these phospholipids are associated with the intact milk fat globule membrane (MFGM). The remaining 35 to 40% are found in the aqueous phase associated with protein/membrane fragment material in solution, rather than still attached to the MFGM (Huang and Kuksis, 1967; Patton and Keenan, 1971).

The MFGM that surrounds the milk fat droplets is derived from the apical plasma membrane of the secretory cells in the lactating mammary glands, and is composed of phospholipids and glycolipids, as well as proteins, glycoproteins, enzymes, triacylglycerols and minor components. Estimates of the proportion of phospholipids in the MFGM vary from 15 to 30%, depending on extraction procedure; however, typical values are at the higher end of the range. For instance, Keenan and Dylewski (1995) reported 26 to 31%, and Norris *et al.* (2003) found 28% of the MFGM as phospholipid (see Keenan and Mather, Chapter 4).



Where the polar group is

Ethanolamine (PE)  
 Inositol (PI)  
 Serine (PS)  
 Choline (PC)

**Figure 1.5.** Fischer projection diagram of a glycerophospholipid showing the stereospecific numbering (*sn*-) convention.

While the polar lipids constitute a very small proportion of the total milk lipids, they play an important role because of their mixed hydrophilic and hydrophobic nature. This unique characteristic of polar lipids is largely responsible for stabilising the suspension of milkfat in the aqueous environment of the milk, allowing the relatively high concentrations of milkfat and protein to coexist in the same solution (Deeth, 1997). In the above process, the major structural features involved are the large non-polar (hydrophobic) fatty acid chains and the polar (hydrophilic) charged head group residue of the phospholipids. The polar lipids contain a variety of polar groups that contribute to the charged nature of the molecules. In addition to the charged head-group, phospholipids contain a negatively charged phosphate group (Figure 1.5).

Dairy phospholipids are important structurally, because they are able to stabilise emulsions and foams, and to form micelles and membranes (Jensen and Newburg, 1995). Phospholipids also have the potential to be pro-oxidants, because they contain mono-unsaturated and poly-unsaturated fatty acids and have the ability to attract metal ions. Phosphatidyl ethanolamine binds copper strongly and is believed to be important in copper-induced oxidation in milk (O'Connor and O'Brien 1995; Deeth, 1997). The polyunsaturated fatty acids and metal ions accelerate lipid oxidation, especially when heat is applied; hence, phospholipids can be degraded during the processing of milk. However, in dairy products, the situation is complex and it appears that phospholipids are able to act as either pro-oxidants or anti-oxidants, depending on the pH, ratio of water and phospholipid species (Chen and Nawar, 1991).

**Table 1.10.** Approximate phospholipid content of different dairy products

Product	Whole milk	Skim milk	Cream (40% fat)	Butter milk
Total fat (% w/w)	4	0.06	40	0.6
Phospholipids (% w/w)	0.035	0.015	0.21	0.13
Ratio (g PL/100g total fat)	0.9	25	0.5	22

Adapted from Mulder and Walstra (1974)

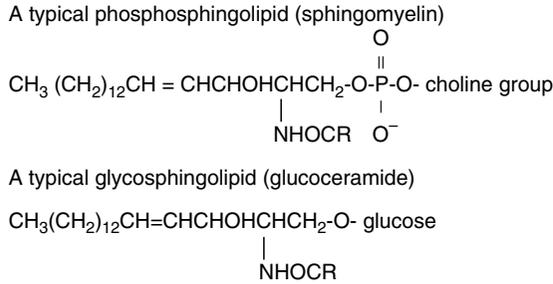
As the milk is processed, the phospholipids are partitioned differently from the neutral lipids (Table 1.10). When the whole milk is separated, the phospholipids tightly bound to the MFGM go into the cream with the neutral lipids, while the phospholipids associated with the protein/membrane fragments in the aqueous phase are retained in the skim milk. Hence, the ratio of phospholipids to total fat is relatively low in cream and high in skim milk. Furthermore, during butter making, a greater proportion of the phospholipids than the neutral lipids from the cream is retained in the buttermilk, leading to a high ratio of phospholipid to the total fat in buttermilk (Table 1.10).

#### 1.4.1. Composition and Structure

The percentage of phospholipids in milk fat is typically within the range 0.5–1.0 %. Bitman and Wood (1990) found that phospholipids in milk tended to decline during lactation, but Kinsella and Houghton (1975) observed little change. While there was a change in the percentage of total phospholipids, the ratio of the major phospholipids remained relatively constant, suggesting a constant ratio of phospholipids in the MFGM.

The structures of the major polar lipids found in the milk are shown in Figures 1.5 and 1.6. Glycerophospholipids [phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylcholine (PC)] have fatty acids at positions *sn*-1 and *sn*-2, and a phosphate and a polar head-group on the *sn*-3 position. Of the minor phospholipids, plasmalogens have a similar structure to phosphatidylcholine and phosphatidylethanolamine but with an ether linkage rather than an ester linkage at the *sn*-1 position. Lysophospholipids have only one fatty acid in the glycerophospholipid.

The sphingophospholipid, sphingomyelin (Sph) consists of a ceramide (a fatty acid linked to a long-chain sphingoloid base through an amide linkage) with a phosphorylcholine headgroup (Figure 1.6). Sphingomyelin



**Figure 1.6.** Typical structures of sphingolipids (phosphosphingolipid and glycosphingolipid classes), based on a d18:1 ceramide (R = fatty acid group).

is generally included in the phospholipid group as it has similar properties (especially with phosphatidylcholine).

Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin are the major polar lipids found in bovine milk and are present in similar proportions in the total phospholipids, about 25 to 35% (Table 1.11). Phosphatidylserine and phosphatidylinositol are present at lower levels, about 3 to 8%. There are also significant amounts of ceramides, as glucoceramide (GluCer, monohexose) and lactoceramide (LacCer, dihexose), 3 to 6%. Table 1.11 shows five sets of analytical data, four of which are relatively

**Table 1.11.** Proportions of individual phospholipids and ceramides in bovine milk (as percentage of total polar lipids)

	a (mol %)	b %(w/w)	c %(w/w)	d %(w/w)	e %(w/w)
Phosphatidylethanolamine	31.8	34.2	31.1	42.0	36.8
Phosphatidylinositol	4.7	6.2	5.2	4.8	na
Phosphatidylserine	3.1	2.8	8.5	6.7	na
Phosphatidylcholine	34.5	25.4	26.4	19.2	32.2
Sphingomyelin	25.2	23.6	28.7	17.9	29.6
Glucoceramide	na <sup>f</sup>	5.0	na	2.7	na
Lactoceramide	na	2.9	na	6.7	na
Phospholipids in milk (mg/10 ml)		2.28	2.51	2.9	2.42

Sources and sampling:

<sup>a</sup> Jensen and Clark (1988); <sup>b</sup> Christie *et al.* (1987), single dairy herd; <sup>c</sup> Bitman and Wood (1990), 12 cows, 42 days of lactation; <sup>d</sup> Rombault *et al.* (2005), farm milk; <sup>e</sup> Fagan and Wijesundra (2004), milk from 21 dairy farms; <sup>f</sup> na not available (not determined)

Note: not all species identified, hence normalisation needs to be carried out prior any to direct comparison

similar. The data indicate variations in the proportions of individual phospholipids, which may depend on the analytical methods used, the number and breed of cows in the sample, and the diet and stage of lactation of cows. Interestingly, the reported total phospholipid content of the milk was similar (~2.5 mg/10 mL milk).

Table 1.12 shows the fatty acid composition of the phospholipids of milk samples from 12 cows (Bitman and Wood, 1990). The major fatty acids of the glycerophospholipids are 18:1, 16:0, 18:0 and 18:2. None of the phospholipids has significant amounts of fatty acids below 14:0 (unlike the

**Table 1.12.** Fatty acid composition of phospholipids from bovine milk

Fatty acid	Fatty acid composition (% w/w)					
	Phosphatidylethanolamine <sup>a</sup>	Phosphatidylinositol <sup>a</sup>	Phosphatidylserine <sup>a</sup>	Phosphatidylcholine <sup>a</sup>	Sphingomyelin <sup>a</sup>	Sphingomyelin (2) <sup>b</sup>
14:0	1.0	1.2	1.0	7.1	4.1	1.8
15:0	0.5	0.5	0.4	1.5	1.1	0.3
16:0	11.4	7.6	8.4	32.2	36.1	18.0
16:1	2.7	2.1	2.2	3.4	0.6	0.6
17:0	0.9	0.9	1.0	1.0	1.5	0.5
18:0	10.3	22.7	25.7	7.5	8.7	4.1
18:1	47.0	35.9	35.5	30.1	1.5	4.5
18:2	13.5	9.5	9.7	8.9	0.2	0.8
18:3	2.3	2.2	2.1	1.4		
20:0	0.6	1.5	1.7	0.4	1.2	0.6
20:3	1.7	4.1	3.1	1.0		
20:4	2.7	5.0	1.8	1.2		
20:5	1.0	1.0	0.8	0.5		
21:0	0.5	1.1	1.2	0.3	1.5	0.8
22:0					14.4	15.9
22:4	0.8	1.1	1.1	0.4		
22:5 n-6	0.6	0.9	0.9	0.4		
22:5 n-3	1.0	1.3	1.7	0.3		
22:6	0.1	0.2	0.2	0.0		
23:0					17.4	30.4
24:0					11.3	17.3
Polyunsaturated	23.7	25.3	21.4	14.1	0.2	0.8
Saturated	26.4	36.7	40.6	51.6	97.6	90.6
% of total	31.1	5.2	8.5	26.4	28.7	—
Phospholipids						

<sup>a</sup> Bitman and Wood (1990), 12 cows, 42 days of lactation

<sup>b</sup> Morrison *et al.* (1965) example of the variation

triacylglycerols). For identification of glycerophospholipids, electrospray ionisation mass spectrometry (ESI-MS) has been described by Kerwin *et al.* (1994) and Myher and Kuksis (1995). Such methods have enabled the identification of the head-groups; hence, the identification of phospholipid species and the fatty acid constituents. Furthermore, these methods can identify the two fatty acids on the same phospholipid.

Sphingomyelin has a unique composition of fatty acids compared to the other phospholipids (Table 1.12) as the fatty acids are mainly long-chain saturated (i.e., 16:0, 22:0, 23:0 and 24:0). To illustrate the differences in fatty acid composition of sphingomyelin, the fatty acid analysis from another study is also listed. Furthermore, Bitman and Wood (1990) and Nyberg (1995) found that the ratio of 16:0 to very long-chain fatty acids is relatively high, while Morrison *et al.* (1965) and Ramstedt *et al.* (1999) found a lower ratio. The major sphingoloid base of sphingomyelin is reported to be sphingosine, a dihydroxy 18C amino alcohol (d18:1) (which introduces unsaturation into the molecule) while d16:1, d16:0 and d18:0 are found to be the minor sphingoloid bases (Ramstedt *et al.*, 1999). Karlsson *et al.* (1998), using HPLC/MS with electrospray and atmospheric pressure chemical ionisation on sphingomyelin, found the same sphingoloid bases as Ramstedt *et al.* (1999).

The specific phospholipids differ in the degree of unsaturation (Table 1.12). Phosphatidylethanolamine has a low content (~26%) of saturated fatty acids, and has especially a high content of linoleic acid, far higher than found in triacylglycerols of milk fat. Phosphatidylinositol and phosphatidylserine have 37–40% saturated fatty acids, whereas phosphatidylcholine has over 50%. The fatty acids in sphingomyelin are almost all completely saturated.

The positional distribution of fatty acids in phosphatidylcholine and phosphatidylethanolamine, the major glycerophospholipids, of bovine milk, were investigated by Morrison *et al.* (1965) and are shown in Table 1.13. Unlike triacylglycerols, phospholipids do not contain short-chain fatty acids (14:0 being the shortest chain fatty acid present at a significant level). This is probably due to differences in the route of synthesis, as most short-chain fatty acids are found at the *sn*-3 position of triacylglycerols, which in phospholipids is occupied by the phosphate moiety. In phospholipids, the polyunsaturated fatty acids tend to be esterified preferentially at the *sn*-2 position, while the saturated fatty acids show a preference for the *sn*-1 position (Table 1.13). For phosphatidylethanolamine, 18:2 and 18:3 are found predominantly at the *sn*-2 position, while 18:1 is fairly evenly distributed and 16:0 and 18:0 are predominantly at the *sn*-1 position. In phosphatidylcholine, which is more saturated than phosphatidylethanolamine, the distribution of saturated and unsaturated fatty acids is less distinct between

**Table 1.13.** Distribution of fatty acids at positions *sn*-1 and *sn*-2 of phosphatidylcholine and phosphatidylethanolamine from bovine and human milks<sup>a</sup>

Fatty acid	Fatty acid composition (mol %)							
	Bovine <sup>b</sup>				Human <sup>c</sup>			
	Phosphatidyl choline		Phosphatidylethanol amine		Phosphatidyl choline		Phosphatidylethanol amine	
	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -1	<i>sn</i> -2
14:0	5.6	10.8	1.9	1.3	3.4	4.9	1.0	1.0
16:0	41.9	30.6	19.7	4.7	34.2	32.3	9.3	8.2
16:1	0.6	1.2	1.2	2.2	1.5	2.2	1.8	3.3
18:0	17.5	2.4	19.0	1.3	34.9	2.1	65.4	1.3
18:1	20.3	27.8	45.8	47.8	14.3	13.7	18.1	15.3
18:2	2.7	9.2	2.9	21.4	2.7	30.9	4.4	30.2
18:3	0.8	1.8	1.1	4.5	–	2.0	–	5.1
20:3	–	1.6	0.2	2.2	–	3.9	–	5.4
20:4	0.2	1.2	0.2	3.0	–	6.6	–	20.9
22:6	–	–	–	–	–	0.8	–	5.2

<sup>a</sup> Christie (1995)<sup>b</sup> Morrison *et al.* (1965)<sup>c</sup> Morrison and Smith (1967)

the *sn*-1 and *sn*-2 positions, though 18:0 and the polyunsaturated fatty acids still show the preference described above.

#### 1.4.2. Ceramides and Gangliosides

The glyceramides (glycosphingolipids) have one or more hexose sugar units attached at position 1 of the ceramide (Figure 1.6), rather than the phosphorylcholine group which is present in sphingomyelin. The concentration of hexose ceramides in polar lipids, as determined by groups of authors, is about 8% (Table 1.11). The fatty acid composition of the ceramides has been reported by Christie (1995), and tends to follow the fatty acid trends found in sphingomyelin.

The gangliosides are complex ceramide polyhexosides, which contain one or more acidic sugars [known as sialic acid or *N*-acetylneuramic acid (NANA)]. The specific names of the gangliosides identify their structure. The letter G followed by M, D, T or Q designates mono-, di-, tri- or tetra-sialic acid groups. The carbohydrate sequence that is attached to the ceramide is designated by a number (5-*n*), where *n* is the number of neutral sugar residues. For example, GD3 is a ganglioside with two NANA units and two neutral sugar residues.

A number of gangliosides, namely GM3, GM2, GM1, GD3, GD2, and GD1, have been isolated from bovine milk, (Keenan and Dylewski, 1995), the major ones being GD3 (~50%) and GM3 (~20%). Martin *et al.* (2001) investigated the seasonal variation in the total gangliosides [measured as lipid-bound sialic acid (LBSA)]. They found that the level of gangliosides was high in the colostrum, which decreased through transitional and mature milk, before rising a little in late lactation milk; mean values were 3.5, 1.3, 0.9 and 1.9  $\mu\text{g/g}$  milk, respectively. Puente *et al.* (1992), who measured the seasonal trend in individual gangliosides, showed that GD3 was slightly higher in colostrum and late-lactation milk compared to mid-lactation milk, while GM3 showed the reverse trend.

The major fatty acids of GD3 and GM3 (the major gangliosides of bovine milk) are 22:0, 23:0, 24:0 and 16:0, with both species of ganglioside having similar fatty acid compositions (Table 1.14). These fatty acids are also a feature of the sphingomyelin structure. Martin *et al.* (2001), who

**Table 1.14.** Fatty acid composition of bovine and human milk gangliosides GD3 and GM3<sup>a</sup>

Fatty acid	Fatty acid composition (% w/w)			
	Bovine milk		Human milk	
	GD3	GM3	GD3	GM3
12:0	0.4	0.5	2.7	1.1
14:0	1.0	1.6	3.8	1.8
15:0	0.3	0.5	1.0	0.6
16:0	10.3	11.9	18.4	11.7
16:1	0.1	0.4	1.4	1.3
17:0	0.3	0.3	0.3	0.4
18:0	4.6	5.7	18.7	16.6
18:1	2.4	4.1	3.8	6.0
18:2	1.2	0.8	2.6	4.2
18:3	0.3	0.4	1.2	0.8
19:0	0.1	0.1	11.1	3.1
20:0	1.5	1.6	5.6	9.5
21:0	1.4	1.3	0.2	0.6
22:0	25.6	23.9	10.9	18.6
22:1	0.9	1.1	1.1	0.8
23:0	26.7	24.1	1.8	2.9
24:0	18.9	17.2	7.7	11.1
24:1	2.8	3.1	6.8	7.6
25:0	1.1	1.0	0	0

<sup>a</sup> Adapted from Bode *et al.* (2004)

investigated the seasonal variation in the fatty acids of the gangliosides, found that the fatty acids were very highly saturated (97%) in colostrum (2 days *post-partum*), while they were much less saturated (68%) in transitional (15 day) milk. Subsequently, the level of saturation increased (78%) in mature (90 day) milk and a further increase was observed (83%) in late lactation milk (10th month).

**1.4.3. Health Issues**

In addition to their importance in cell membranes and in cell signaling, specific polar lipids are recognized to have a number of positive health effects relating to immune function, heart health, brain health and cancer. These effects are related to either the polar lipids themselves or to their metabolites (Vesper *et al.*, 1999). Sphingomyelin, plasmalogen and ceramides have shown strong anti-tumour activity. Sphingomyelin can influence cholesterol metabolism and coronary heart disease. Sphingomyelin and gangliosides exhibit anti-infection activity. Phospholipids may also protect against mucosal damage. Parodi (2004; Chapter 13) reviewed the subject matter of polar lipids in human nutrition and Ward *et al.* (Chapter 6) also discussed polar lipids in milk.

**1.5. Minor Constituents**

**1.5.1. Sterols**

Sterols are minor components of milk lipids, which make up just 0.3% of the total fat (Table 1.1). The principal component is cholesterol, which accounts for over 95% of the total; about 10% of the cholesterol is present in the esterified form. Small amounts of other sterols, namely campesterol, stigmasterol and  $\beta$ -sitosterol, have also been identified in milk fat (Mincione *et al.*, 1977).

The cholesterol content, expressed as mg/g fat, of dairy products varies considerably (Table 1.15). Cholesterol values for skim milk (~44 mg/g fat) and buttermilk (~8.5 mg/g fat) are much higher than the normal value of

**Table 1.15.** Cholesterol content of different dairy products<sup>a</sup>

	Whole milk	Cream	Skim milk	Butter	Buttermilk
Fat content (% w/w)	4.7	43.0	0.06	82.5	1.3
Cholesterol (mg/g fat)	3.3	3.1	44	2.8	8.5

<sup>a</sup> Russell and Gray (1979)

cholesterol (3 mg/g fat) found in milk. As a result of churning, buttermilk contains a considerable quantity of MFGM material and a relatively high concentration of small fat globules. Similarly, during separation, small fat globules and membrane material are concentrated in skim milk. Since small fat globules have a greater amount of membrane material relative to the amount of fat in the core as compared to large fat globules, it can be concluded from the above data that the amount of cholesterol (mg/g fat) is greater in the membrane material than in the fat core of the globule.

### 1.5.2. Carotenoids

The principal pigment in milk fat is  $\beta$ -carotene, accounting for about 95% of the total carotenoids present. In milk,  $\beta$ -carotene is found in the core of the milk fat globules and is absent from the membrane (Jensen and Nielsen, 1996).

The concentration of  $\beta$ -carotene in milk depends on the level of  $\beta$ -carotene in feed and on the breed of cow. Carotenoid pigments are particularly high in fresh grass, but substantially lower in a mix of concentrate and hay, a normal winter feed. Channel Island cows, Jerseys and Guernseys, have a higher level of  $\beta$ -carotene and a lower level of vitamin A in their milkfat than other breeds, such as Friesians. In New Zealand, cows fed fresh spring grass can have  $\beta$ -carotene up to 13  $\mu\text{g/g}$  fat (Jersey) or 8  $\mu\text{g/g}$  fat (Friesian) in their milk. However, when cows are grazed on mature summer pasture, these values decrease substantially to about 7 and 4  $\mu\text{g/g}$  fat for Jersey and Friesian, respectively (Winkelman *et al.*, 1999). In view of these variations, it is not surprising that value for  $\beta$ -carotene in commercial butters ranges from 2.5 to 12.5  $\mu\text{g/g}$  fat (Buss *et al.*, 1984).

### 1.5.3. Fat-soluble Vitamins

A significant nutritional aspect of milk lipids is that vitamins A, D, E and K are dissolved in the fat phase. Milk fat is considered to be a significant source of vitamin A, but a poor source of vitamins D and K (McBean and Speckmann, 1988).

Vitamin A is a fat-soluble vitamin involved in critical biological functions, such as embryonic development, growth and vision. It has three primary forms; retinol, retinal and retinoic acid. In addition,  $\beta$ -carotene can be converted to some extent in the body into retinol and is therefore called provitamin A. The bioactivity of these vitamin A compounds varies considerably, ranging from 100% for all-*trans* retinol, 75% for 13-*cis* retinol and to just 17% for  $\beta$ -carotene. All-*trans* retinol is the major form of vitamin A in milk fat, with values ranging from 8.0 to 12.0  $\mu\text{g/g}$  fat in samples of commercial butter. In contrast, 13-*cis* retinol is present at a very low

concentration, 0.5 to 1.5  $\mu\text{g/g}$  fat (Buss *et al.*, 1984). The total vitamin A bioactivity can be obtained by summation of the concentrations and activities of the different forms of vitamin A. In milk fat, this gives an average value of approximately 12 retinol equivalents/g fat or 40 IU/g fat.

Vitamin E is an effective scavenger of lipid peroxy radicals and is efficient at protecting unsaturated fatty acids against lipid peroxidation. The chemistry of vitamin E is rather complex as there are eight compounds, four tocopherols and four tocotrienols, which exhibit vitamin E activity. The relative bioactivity of the various compounds varies considerably, from 1.0 for  $\alpha$ -tocopherol to 0.03 for  $\delta$ -tocopherol. In milk,  $\alpha$ -tocopherol accounts for virtually all of vitamin E, although very small amounts of  $\beta$ -tocopherols and  $\gamma$ -tocopherols are present. Also, the concentration of  $\alpha$ -tocopherol in milk fat varies widely, with the level in samples of commercial butter ranging from 18 to 35  $\mu\text{g/g}$  fat (MAFF, 1999). These concentrations equate to a low level of vitamin E bioactivity, 0.025 to 0.05 IU/g fat.

The level of vitamin D in milk fat is very low, about 0.01 to 0.02  $\mu\text{g/g}$  fat, which equates to 0.4 to 0.8 IU/g fat. Vitamin K is also present in milk at a very low concentration. The level of phyloquinone (vitamin K<sub>1</sub>) is reported to be 0.1 to 0.2  $\mu\text{g/g}$  fat (Haroon *et al.*, 1982).

#### 1.5.4. Flavour Compounds

The chemistry of the flavour of milk fat and butter is very complex, involving a large number of compounds contributing to the overall aroma and taste. Approximately 200 volatile compounds have been identified in milk fat (Schieberle *et al.*, 1993). However, many of the volatile compounds are present at concentrations below their individual flavour threshold level, and the extent to which these compounds contribute to the overall flavour profile is not known fully. The perceived flavour of milk fat can be altered by a change in the concentration of individual volatile compounds. The principal factor that can change the concentration of the volatile compounds is the feeding regime of the cow (Bendall, 2001).

The main classes of volatile compounds, which are considered to contribute significantly to the overall flavour are lactones, fatty acids, aldehydes and methyl ketones. As noted earlier, there are very small amounts of hydroxy acids, esterified to triacylglycerols, in milk fat. These act as precursors of flavoursome  $\gamma$ -lactones and  $\delta$ -lactones. It has been reported that three lactones,  $\delta$ -octalactone,  $\delta$ -decalactone and  $\gamma$ -dodecalactone, are important flavour components in milk fat (Widder *et al.*, 1991; Schieberle *et al.*, 1993). Siek *et al.* (1969) identified the short-chain fatty acids, 4:0 and 6:0, as key flavour components of milk fat. However, while they may contribute to the overall flavour of milk fat at very low concentrations,

higher levels caused by lipolysis will inevitably lead to undesirable rancid flavours. Aliphatic aldehydes are potentially major contributors to both desirable and undesirable flavours of milk fat. At very low concentrations (i.e., at or below their flavour threshold values), some aldehydes are thought to contribute desirable flavours. For example, hept-4-enal at 0.001  $\mu\text{g/g}$  fat is claimed to contribute a creamy flavour to butter (Urbach, 1979). However, slight oxidation of milk fat can increase the concentrations of aldehydes to levels at which undesirable oxidized flavours predominate. Fresh milk fat contains virtually no methyl ketones. However, on heating, the trace amounts of  $\beta$ -keto acids present in milk fat are decarboxylated to yield methyl ketones. These volatiles are considered to contribute the rich flavour associated with baked goods that contain butter. In addition to the four classes of compounds discussed above, there are several other volatile compounds which contribute to the aroma and taste of milk fat, including diacetyl and skatole.

## 1.6. Milk Fat From Different Animal Species

A wide range of animals, large or small, living on land or in water, surviving in hot or cold climates produce milk. Inevitably there are variations in the composition of milk from these animals. Some of these variations are within species caused by factors, such as region, stage of lactation and season, as discussed earlier. However, when comparing different species, additional factors need to be considered that relate to the physiology of the animals, such as whether the animal is a ruminant or a non-ruminant; suckling frequency; development factors, such as growth rate; and environmental factors, such as temperature.

The detailed composition of milk from a wide range of mammals has been reviewed by IDF (1986), Alston-Mills (1995), Iverson and Oftedal (1995), Oftedal and Iverson (1995), IDF (1996) and Elagamy (2003). In the current discussion, the focus is on milks that are consumed by humans, though other examples have been included by way of comparison. Obviously, the variations within a species mean that quoted values are intended only to be indicative of the typical concentrations rather than definitive values.

### 1.6.1. Gross Composition

The range of fat content in the milk of different animal species varies widely (Table 1.16). The milk of the cow, human, goat and camel have a low fat content. The milk of the buffalo and sheep have values that are a little higher, whereas the fat content of the milk of the rabbit and seal is much

**Table 1.16.** Average fat content of milk from various species

	Fat content (% w/v)	References
Cow	3.5	Elagamy (2003)
Sheep	7.8	Elagamy (2003)
Goat	4.2	Elagamy (2003)
Buffalo	6.8	Elagamy (2003)
Camel	4.0	Elagamy (2003)
Rabbit	18.3	Jeness and Sloan (1970)
Seal	42.1	Iverson <i>et al.</i> (1997)
Pig	6.8	Mellies <i>et al.</i> (1978)
Human	3.8	Jensen (1988)

higher. No distinct relationship is observed between the fat content of milk of ruminants (cow, sheep, goat, buffalo and camel) and that of non-ruminants (human, rabbit, seal and pig). However, domesticated animals tend to produce milk that is lower in fat content than non-domesticated animals (Oftedal and Iverson, 1995).

Low suckling frequency and a high metabolic rate have been associated with a high fat content. It has been suggested that the high fat content of rabbit's milk is due to the infrequent suckling of the young, about once a day (Oftedal and Iverson, 1995). Marine mammals may have a high fat content in their milk to facilitate fat deposition in the suckling young and, thus, to reduce heat loss. In addition, a high solids content reduces the water requirement in the milk, which could be advantageous when the water has to come from the body reserves of the mother. The major lipid class in the milk of animals is triacylglycerols, and this usually accounts for 97–98% of the total lipids (Christie, 1995).

### 1.6.2. Fatty Acids

The fatty acids in milk fat are derived from two sources, *de novo* synthesis of fatty acids in the mammary gland and plasma lipids (see Palquist, Chapter 2). *De novo* synthesis generally involves short-chain and medium-chain fatty acids and some 16:0. The proportions of various fatty acids depend on the specific balance between enzymatic chain elongation and chain termination. The plasma lipids are derived from the diet and also from storage in the body tissues. For non-ruminants, the diet has a large influence on the fatty acid composition but for ruminants, biohydrogenation in the rumen results in much less impact of diet on the final fatty acids absorbed into the bloodstream.

One distinct difference between the fatty acid composition of the various species is the proportion of short-chain fatty acids in the milk of

**Table 1.17.** Major fatty acids in milk triacylglycerols from various species

Fatty acid	Fatty acid composition (% w/w)								
	Cow	Sheep	Goat	Buffalo	Camel	Rabbit	Fur Seal	Pig	Human
4:0	3.9	4.0	3.1	3.6	1.0				0.2
6:0	2.5	2.8	2.2	1.6					0.2
8:0	1.5	2.7	2.4	1.1	0.5	22.4			0.5
10:0	3.2	9.0	6.3	1.9	0.1	20.1		0.7	1.0
12:0	3.6	5.4	2.9	2.0	0.5	2.9	0.2	0.5	4.4
14:0	11.1	11.8	7.7	8.7	10.0	1.7	8.7	4.0	6.3
14:1	0.8				1.5		0.4		
15:0	1.2						0.3		0.4
16:0	27.9	25.4	22.0	30.4	31.5	14.2	18.5	32.9	22.0
16:1	1.5	3.4	1.9	3.4	9.0	2.0	10.0	11.3	3.7
18:0	12.2	9.0	10.6	10.1	14.0	3.8	1.8	3.5	8.1
18:1	21.1	20.0	23.7	28.7	25.0	13.6	22.5	35.2	34.0
18:2	1.4	2.1	2.7	2.5	3.0	14.0	1.4	11.9	10.9
18:3	1.0	1.4	1.0	2.5		4.4	0.8	0.7	0.3
>C <sub>19</sub>							29.4		

Cow, (Creamer and MacGibbon, 1996); Sheep, (Glass *et al.*, 1967); Goat, (Posati and Orr, 1976); Buffalo, (Glass *et al.*, 1967); Camel, (Gnan and Sheriha, 1981); Rabbit, (Glass *et al.*, 1967); Antarctic fur seal, (Iverson *et al.*, 1997); Pig, (Glass *et al.*, 1967); Human, (Jensen *et al.*, 1995).

cow, sheep, goat and buffalo compared to the milk of camel, rabbit, fur seal, pig and human (Table 1.17). Interestingly, the milk of the camel (a ruminant) contains little C<sub>4</sub> to C<sub>12</sub> fatty acids, whereas the rabbit (a non-ruminant) has over 40% of the fatty acids as the medium-chain length fatty acids, C<sub>8</sub> to C<sub>10</sub>.

Generally, the milk fat of non-ruminants has a higher level of polyunsaturated fatty acids than the milk fat of ruminants, due to the direct absorption of these fatty acids from the diet (Table 1.17). Marine mammals, such as the fur seal, have also high levels of long-chain polyunsaturated fatty acids, 20:5 and 22:6, due to the presence of these fatty acids in the diet (Iverson *et al.*, 1997).

Precht *et al.* (2001) found that the proportion of *trans*-18:1 fatty acid isomers was similar in the milk fat of the cow, goat and sheep: vaccenic acid (11*t*-18:1) was the major isomer in all these milks, accounting for 51, 37 and 47% of the total, respectively. The isomers between 9*t* and 16*t*, excluding the 11*t*, made up most of the rest (4–10% each).

Methods for distinguishing cow, sheep and goat milk, using the differences in fatty acid composition, have been reviewed by Ramos and Juarez (1986). These methods involve comparing the ratios of fatty acids. For example, the 12:0/10:0 fatty acid ratio is consistently higher in cow's milk (0.9–1.3) than in sheep's milk (0.4–0.8) or goat's milk (0.3–0.4)

(Bernassi, 1963; Ramos and Juarez, 1986). Other ratios that have been considered include 14:0/12:0 and 14:0/8:0, and more complex combinations, such as 10:0/(12:0 + 16:0 + 18:1).

**1.6.3. Triacylglycerols**

Triacylglycerols are synthesised in the mammary gland, and the enzyme specificity and substrate availability play a role in the final structure of the molecule. Table 1.18 shows the fatty acids esterified at each position of the triacylglycerols in the milk of various species. The distinctive feature of the triacylglycerols of ruminants (cow, sheep and goat) is the presence of the short-chain fatty acids, 4:0 and 6:0, which are esterified almost exclusively at the *sn*-3 position. In human and porcine milk fat more than 50% of the 16:0 is at the *sn*-2 position; however, in the milk fat of cow, sheep and goat, 16:0 is distributed more evenly between the *sn*-1 and *sn*-2 positions. 18:1 is esterified preferentially at the *sn*-1 and *sn*-3 positions in human, porcine and bovine milk fat, although for the cow, the total amount of 18:1 is less. In ovine and caprine milk fat, 18:1 is favoured at the *sn*-3 position and there are smaller but similar amounts at the other two positions.

Ruiz-Sala *et al.* (1996) compared the triacylglycerol composition of the milkfat of sheep, cow and goat by HPLC, using ELSD detection. They

**Table 1.18.** Fatty acids esterified at each position of the triacylglycerols in the milks from various species

Fatty acid composition (mol % of total fatty acids)															
Cow			Sheep			Goat			Pig			Human			
<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3	<i>sn</i> -1	<i>sn</i> -2	<i>sn</i> -3	
4:0	0.4	30.6			10.8			13.2							
6:0	0.7	13.8			10.4			10.6							
8:0	0.3	3.5	4.2	0.3	2.0	4.4	1.7	1.2	4.6						
10:0	1.4	8.1	7.5	1.4	5.2	10.3	3.3	6.9	12.2			0.2	0.2	1.1	
12:0	3.5	9.5	4.5	2.2	4.7	3.5	4.0	4.6	1.2			1.3	2.1	5.6	
14:0	13.1	25.6	6.9	8.2	17.6	5.3	8.4	20.3	2.7	2.4	6.8	3.7	3.2	7.3	6.9
16:0	43.8	38.9	9.3	38.0	23.8	2.5	43.6	33.9	3.4	21.8	57.6	15.4	16.1	58.2	5.5
18:0	17.6	4.6	6.0	19.1	12.6	9.1	15.3	6.3	7.7	6.9	1.1	1.4	15.0	3.3	1.8
18:1	19.7	8.4	17.1	18.7	19.3	27.2	16.1	16.1	30.2	49.6	13.9	51.7	46.1	12.7	50.4
18:2				2.7	4.2	6.0	0.3	2.5	4.5	11.3	8.4	11.5	11.0	7.3	15.0
18:3				2.2	1.7	4.4				1.4	1.0	1.8	0.4	0.6	1.3

Cow, (Parodi, 1979); Sheep and Goat, (Kuksis *et al.*, 1973); Pig, (Christie and Moore, 1970); Human, (Breckenridge *et al.*, 1969).

found significant differences in the proportions of very low molecular weight triacylglycerols (carbon number  $\leq 34$ ) in the three milk fats, with sheep having the highest proportion (18.2%), followed by goat (15.2%) and cow (10.8%). This supports the earlier work of the same group (Barron *et al.*, 1990) using HPLC and UV detection. Andreotti *et al.* (2002) used  $^{13}\text{C}$  nuclear magnetic resonance (NMR) to study the low molecular weight triacylglycerols in cow, sheep and goat milk fats. Principal component analysis of the signals from multiple samples showed distinct clusters relating to each of the three sources of milk fat, providing a possible method for distinguishing between the milks of these three species.

Precht (1992) developed a method for determining foreign fats in cows' milk using a mathematical combination for the levels of specific individual triacylglycerols. This formed the basis of a European standard method for detecting foreign fats in bovine milk fat (European Commission, 1999). The limit of detection of adulteration depends on the foreign fat, but is typically 2–7%. Goudjil *et al.* (2003) and Fontecha *et al.* (1998) used a modification of the Precht method to detect non-milk fats in sheep and goat milks. While effective for non-milk fats, the methods are less sensitive in distinguishing adulteration of one milk fat with another. For instance, they were able to detect only 30% of cow's milk in sheep's milk.

#### 1.6.4. Polar Lipids

The proportions of corresponding phospholipid classes in the milk of various animal species are remarkably similar (Table 1.19). In each case, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin are the

**Table 1.19.** Phospholipid classes in the milk from various animal species

Species	Composition (mol % of total phospholipids)				
	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Phosphatidyl-serine	Phosphatidyl-choline	Sphingomyelin
Cow <sup>a</sup>	32	5	3	35	25
Sheep <sup>a</sup>	36	3	3	29	28
Goat <sup>b</sup>	33	6	7	26	28
Buffalo <sup>a</sup>	30	4	4	28	32
Camel <sup>a</sup>	36	6	5	24	28
Ass <sup>a</sup>	32	4	4	26	34
Pig <sup>a</sup>	37	3	3	22	35
Human <sup>a</sup>	26	4	6	28	31

<sup>a</sup> Morrison (1968)

<sup>b</sup> Kataoka and Nakae (1973)

major phospholipids, with smaller amounts of phosphatidylinositol and phosphatidylserine. Morrison (1968) reiterated the fact that the total proportion of choline-containing phospholipids (phosphatidylcholine and sphingomyelin) is remarkably constant; the total for the camel is 52% and for all the others is in the range 56–61%, suggesting that these phospholipids perform a similar function in all milks.

The positional distribution of the fatty acids in two phospholipids, phosphatidylcholine and phosphatidylethanolamine, in bovine and human milk is described in Table 1.13 (Christie, 1995). The fatty acids in bovine milk phospholipids have a lower degree of unsaturation than those in human milk phospholipids. However, the distribution of fatty acids between the two positions is similar. Polyunsaturated fatty acids are attached preferentially at the *sn*-2 position, the monounsaturates are distributed similarly between the two positions, and 18:0 is esterified preferentially at the *sn*-1 position; while the other saturated fatty acids (14:0, 16:0) are distributed more evenly (bovine phosphatidylethanolamine being an exception). The phospholipids in human milk show remarkable selectivity for 18:0 at the *sn*-1 and 18:2 at the *sn*-2 positions.

Pan and Izumi (2000) found that the concentration of gangliosides in human milk (9.5 mg LBSA/L) is higher than in cows' milk (4.0 mg LBSA/L). The gangliosides in human and bovine milk also differ in their fatty acid composition (Bode *et al.*, 2004). GD3 and GM3 for each species had similar fatty acid composition, but differences existed between the species (Table 1.14). Human milk gangliosides contain less very long-chain saturated fatty acids, especially 23:0, than bovine milk gangliosides, and more of 16:0 and 18:0. It is not known whether these differences have any influence on the physiological effects of the gangliosides.

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# Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon

D.L. Palmquist

## Abstract

Ruminant milk fat is of unique composition among terrestrial mammals, due to its great diversity of component fatty acids. The diversity arises from the effects of ruminal biohydrogenation on dietary unsaturated fatty acids and the range of fatty acids synthesized *de novo* in the mammary gland.

Forty to sixty per cent of milk fatty acids are long-chain (predominantly C<sub>18</sub>) fatty acids derived from the diet, dependent on the amount of fat in the diet. Fatty acids from C<sub>4</sub> to C<sub>14</sub> are synthesized *de novo* in the mammary gland whereas C<sub>16</sub> arises from both diet and *de novo* synthesis.

Milk fat is the most variable component of milk, both in concentration and composition. In dairy cattle, both the concentration and composition of milk fat are influenced by the diet. Concentration is reduced by feeding diets that contain large proportions of readily-fermentable carbohydrates (starch) and unsaturated fat. Conversely, the percentage of fat in milk can be increased by feeding rumen-inert fats. In ruminants, in contrast with non-ruminants, dietary fats have little effect on milk fat composition. Nevertheless, subtle changes in composition and manufacturing functionality can be effected by feeding different fats. Those fatty acids synthesized *de novo*, especially C<sub>12</sub> to C<sub>16</sub>, and oleic acid (C<sub>18:1</sub>) show greatest variation when supplemental fats are fed.

Modern developments in the manufacture of rumen-protected and rumen-inert fats, together with increased understanding of ruminal and

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

animal lipid metabolism, provide considerable flexibility in manipulation of the composition of milk fat for specific nutritional and manufacturing needs.

Future advances in the science of milk fat and nutrition will come from focusing on the unique biological properties of minor milk fatty acids arising from ruminal biohydrogenation and possibly some of *de novo* mammary origin.

## 2.1. Introduction

Among the biological lipids, few exceed bovine milk fat in the complexity of fatty acids present and triacylglycerol (TAG) structure. This, together with its importance commercially as a human food, has generated very large data bases on the synthesis and composition of milk fat. In spite of this, Jensen (2002) lamented the paucity of new information on the content of trace fatty acids and complex lipids in milk fat.

If one considers the review of Jensen (2002) to represent current knowledge of the composition of bovine milk lipids, it is instructive to consider the review of Garton (1963) as a point to begin the topic of this chapter. Garton (1963) quoted a study reporting 64 individual fatty acids in milk fat; further on, he marvelled at the “bewildering complexity of unsaturated fatty acids present in the milk fat of ruminants,” noting that these were apparently associated with ruminal microbial metabolism, in particular ruminal biohydrogenation. Jensen (2002) lists 416 fatty acids in bovine milk lipids. Forty years ago, the complexity of bovine lipids was recognized, as well as the dual sources of origin, i.e., *de novo* synthesis of the short-chain fatty acids in the mammary glands and longer-chain fatty acids of dietary origin. However, little was known of the quantitative significance of each. Rapid progress in studies of fatty acid synthesis in the mammary glands occurred between 1960 and 1980. More recent efforts have been directed toward characterizing genetic determinants of, and the physiological regulation of, lipid synthesis and evaluating the effects of milk fatty acids on human health and as unique regulators of cell and gene function.

As knowledge of the biological functions of minor milk fatty acids has increased, a new appreciation for studies of effects of nutritional and animal management on milk fat composition has been gained. Because animal nutrition and management are key factors in the manipulation of milk fat composition, this chapter will address the synthesis of milk fatty acids and glycerides from the standpoint of regulation and opportunities for manipulation by various feeding strategies.

## 2.2. Origin of the Fatty Acids in Milk Fat

### 2.2.1. Overview

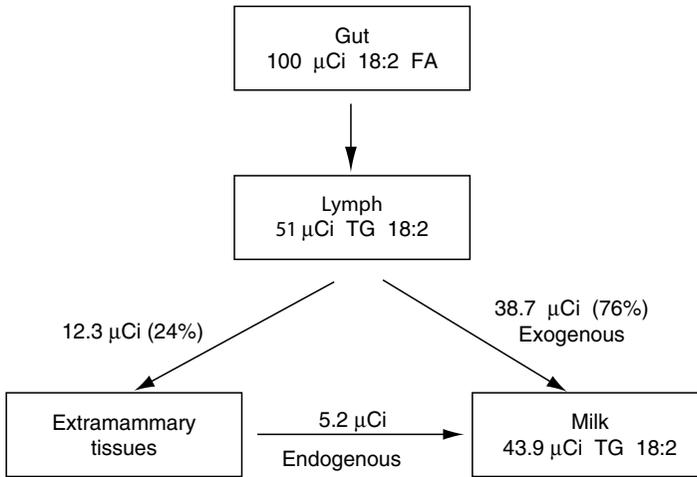
The question of the origins of milk fat, whether wholly from the diet or synthesized by the animal, was an early topic of debate (Jordan and Jenter, 1897). A leading theory, put forth by the eminent lipid chemist, Hilditch (1947), was that the unique short-chain fatty acids arise from the degradation of oleic acid. This conclusion was based upon empirical observations on the composition of ruminant depot and milk fats.

### 2.2.2. Fatty Acid Transport

Modern concepts of milk fat synthesis developed rapidly in the 1950s with the carefully-designed physiological studies in lactating goats by Popják *et al.* (1951) which showed unequivocally the *de novo* synthesis of short-chain fatty acids from  $^{14}\text{C}$ -labelled acetate. Also, the incorporation of tritium-labelled stearic acid into milk fat was demonstrated by Glascock *et al.* (1956). From empirical calculations of the quantity of dietary fat and the recovery of label in milk fat, the latter authors estimated that dietary fat contributed a maximum 25% of the weight of milk fat.

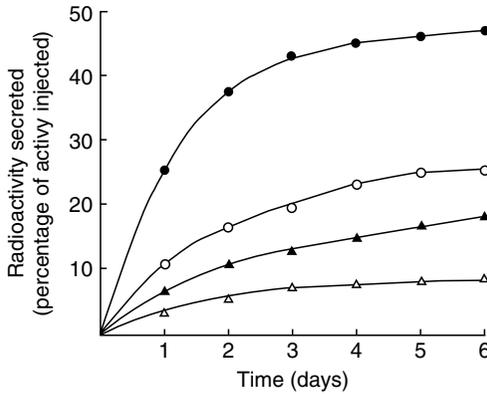
From further studies that compared specific radioactivities of the TAGs in plasma  $\beta$ -lipoproteins and milk fat, Glascock *et al.* (1966) concluded that up to 48% of milk fatty acids were derived from  $\beta$ -lipoprotein TAGs. Because the long-chain fatty acids comprise approximately 50% of milk fat, this observation implied that more than 90% of these fatty acids were of plasma origin, consistent with the demonstration of little fatty acid elongation in the mammary gland (Palmquist *et al.*, 1969). Finally, Glascock and coworkers (see Bishop *et al.*, 1969) concluded, from comparisons of specific radioactivities of dextran sulfate-precipitable lipoproteins and milk fat labelled with tritiated palmitic acid, that no more than 36% of the milk fat could have been derived from the plasma TAGs. In neither study did Glascock and colleagues provide data on quantities of dietary fat nor on the yield and composition of milk fat. However, in the latter study (Bishop *et al.*, 1969), they indicated that palmitic acid constituted 39% of milk fat by weight, a value that would be observed only at a very low dietary fat intake. Thus, the lower contribution of plasma TAGs to milk fat synthesis may not be incompatible with the earlier study (Glascock *et al.*, 1966).

Palmquist and Conrad (1971) fed or intravenously infused 1- $^{14}\text{C}$  palmitic acid into lactating cows and mathematically calculated the partition of  $^{14}\text{C}$  secreted into milk fat as originating from two pools, diluted due to turnover of the dietary and endogenous fatty acids. The proportions of the long-chain fatty acids in milk derived from the diet were influenced by the



**Figure 2.1.** Model of linoleic acid transport from intestine to milk. Incorporation into intestinal lipoprotein triacylglycerols accounted for 51% of absorbed  $^{14}\text{C}$  linoleic acid; of this 76% was incorporated directly into milk whereas 10% was incorporated into milk after cycling through other metabolic pool(s). Adapted from Palmquist and Mattos (1978).

composition of the diet. In further studies, Palmquist and Mattos (1978) injected 1- $^{14}\text{C}$ -linoleic acid as labelled chylomicron TAGs, very low density lipoproteins (VLDL) or albumin-bound fatty acids. From curve analysis of labelled milk fat secretion and reanalysis of earlier data reported by Palmquist and Conrad (1971), they concluded that 88% of long-chain fatty acids in milk were derived directly from TAGs of intestinal lipoproteins while 12% were derived from TAGs of endogenous origin. A model was developed, showing that 76% of absorbed TAG linoleic acid was taken up directly by the mammary glands (Figure 2.1). Using a more direct approach, Glascock *et al.* (1983) injected  $^3\text{H}$  TAGs into sheep at various stages of lactation and measured the partition of label to adipose TAGs, milk lipids or oxidation, as measured by appearance of  $^3\text{H}$  in the body water. Partition of blood TAGs to milk clearly declined with increasing days in lactation (Figure 2.2), with maximal incorporation (46%) at 18 days of lactation. The proportion of injected TAGs transferred to milk fat was much lower than that estimated for lactating cows by Palmquist and Mattos (1978) and the estimated proportion that was oxidized was much higher than reported for lactating goats by Annison *et al.* (1967). Both of these divergent observations of Glascock *et al.* (1983) may be due to use of non-dairy breeds of sheep. Nevertheless, the point of changing partition of plasma TAGs in different physiological states was clearly established, consistent with the principles of homeorhesis described by Bauman and



**Figure 2.2.** Cumulative secretion of radioactivity in milk fat of sheep after intravenous injection of an emulsion of [ $^3\text{H}$ ] triacylglycerol at (●) 18 days, (○) 35 days, (▲) 58 days and (△) 73 days after lambing. (From Glascock *et al.*, 1983; reprinted with the permission of Cambridge University Press).

Currie (1980). Detailed studies by Annison and colleagues (Annison *et al.*, 1967; West *et al.*, 1972) showed that both TAG fatty acids and non-esterified fatty acids (NEFAs) of plasma were taken up by the mammary glands of lactating goats. TAG uptake accounted for up to 60% of milk fat secreted. However, no net uptake of NEFAs occurred. The specific activity of radiolabelled NEFAs decreased across the gland, indicating dilution of NEFAs by fatty acids arising from the hydrolysis of plasma TAGs in the capillaries by mammary lipoprotein lipase (LPL) and subsequent mixing with plasma NEFAs. Similarly, Mendelson and Scow (1972) showed that 40% of TAG fatty acids hydrolyzed by rat mammary LPL were released in plasma, thus mixing with plasma NEFAs. Net uptake of plasma NEFAs by the mammary glands occurs at the high NEFA concentrations (Miller *et al.*, 1991; Nielsen and Jakobsen, 1994) observed immediately *post-partum* or in sub-clinical ketosis. Usually, in established or mid-lactation, net uptake (*ca.* 40  $\mu\text{mol/L}$ ) is variable (+ or -), averaging about zero, so that uptake of fatty acids from the NEFA pool is not evident unless labelled tracers are used. However, milk fat concentration is positively correlated ( $r = 0.76, P < 0.05$ ) with plasma NEFA concentration (Pullen *et al.*, 1989). Significant amounts of NEFAs were transferred from plasma to milk in the mammary glands of lactating rabbits and rats (Jones and Parker, 1978).

### 2.2.3. Lipoprotein Lipase

The TAG-rich chylomicra and VLDL of plasma are the primary source of long-chain fatty acids taken up by the mammary glands. Uptake

is mediated by lipoprotein lipase (LPL), an enzyme that hydrolyzes TAGs to form fatty acids, glycerol and perhaps 2-monoacylglycerol. Mammary gland LPL was described first by Korn (1962) who showed its presence in cows' milk; subsequently, LPL activity was characterized in guinea pig mammary glands by McBride and Korn (1963) and Robinson (1963). The enzyme in tissue and milk is identical (Castberg *et al.*, 1975). Characteristics and regulation of mammary LPL were discussed in detail by Barber *et al.* (1997). LPL is associated with vascular endothelial surfaces, bound by heparin sulfate chains; it is released rapidly by intravenous injection of heparin, which competes with endothelial binding sites of the enzyme, and by the presence of TAG-rich lipoproteins. The enzyme has an absolute requirement for apolipoprotein C-II for activation and is inhibited by apolipoprotein C-III and by 0.5 M NaCl. Apolipoproteins E, which have strong heparin-binding properties, anchor TAG-rich lipoproteins to the endothelial cell walls (Goldberg, 1996). Mammary LPL activity increases markedly immediately prior to parturition and remains high throughout lactation in the guinea pig (McBride and Korn, 1963; Robinson, 1963), rat (Hamosh *et al.*, 1970) and cow (Shirley *et al.*, 1973; Liesman *et al.*, 1988), whereas it is simultaneously down-regulated in adipose tissue (Hamosh *et al.*, 1970; Shirley *et al.*, 1973). These coordinated changes are mediated by the anterior pituitary *via* the release of prolactin (Zinder *et al.*, 1976; Liesman *et al.*, 1988; Thompson, 1992), and nicely illustrate the concept of homeorhetic regulation of physiological function (Bauman and Currie, 1980).

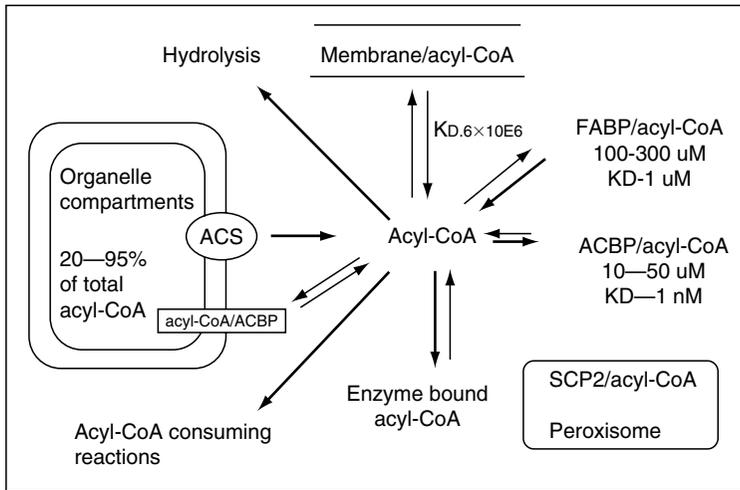
#### **2.2.4. Transport of Long-Chain Fatty Acids into Mammary Cells**

The mechanism(s) for the transfer of fatty acids from the capillaries into mammary cells are not well-documented. Enjalbert *et al.* (1998) observed a nearly linear correlation between mammary arterio-venous difference and arterial concentration of NEFAs + TAGs in the range of 400 to 750  $\mu\text{molar}$  ( $\mu\text{M}$ ), or below saturation for uptake, as described by Baldwin *et al.* (1980). Veercamp *et al.* (1991) suggested that saturation of fatty acid uptake may result from subsequent intracellular processes (see below), from limitations of intracellular diffusion or from diffusive equilibration between extracellular albumin and intracellular fatty acid-binding protein (FABP). A "flip-flop" model, implying carrier-mediated transport, may be too slow to account for fatty acid uptake (Hajri and Abumrad, 2002). Barber *et al.* (1997) have argued for the role of a specific 88 kDa protein, fatty acid translocator (FAT, CD 36), in the transport of fatty acids across the mammary epithelial cell membrane, working in conjunction with intracellular FABP. Scow *et al.* (1980) and Blanchette-Mackie and Amende (1987) made a case for the strongly amphipathic fatty acid molecules to move laterally in a continuous

interface composed of the chylomicron (VLDL) surface film and the external leaflet of plasma and intracellular membranes of endothelial and parenchymal cells, with removal within the cell cytosol as the fatty acids become esterified. Though this model (Scow *et al.*, 1980) is supported by numerous exquisite photomicrographs, it does not explain the loss of up to 40% of the fatty acids from hydrolyzed chylomicron TAGs as plasma NEFAs (Mendelson and Scow, 1972) nor does it provide a role for intracellular FABP. An alternative model is that described for the diffusion of the amphipathic fatty acids through the amphipathic microvillus membrane of the intestine (Thomson *et al.*, 1983). Although diffusion would be too slow for uptake of albumin-bound NEFAs, it could account for uptake from TAG-rich lipoproteins tightly associated with the capillary endothelium (Hajri and Abumrad, 2002). In this model, longer-chain and more saturated fatty acids diffuse through the membrane more rapidly because they are more hydrophobic, consistent with the order of uptake of fatty acids by the bovine mammary gland (Thompson and Christie, 1991). Because the concentration of CoASH is very low and well below saturation in cytosol, it could well be that the limiting steps in the rate of fatty acid uptake are those that determine the rate of fatty acyl-CoA incorporation into TAGs which, in turn, frees up CoASH for acyl CoA synthetase, thereby freeing up a site for binding of a new fatty acid to otherwise saturated FABP and subsequent removal of another fatty acid from plasma; e.g., if there are no intracellular binding sites available for fatty acids from TAG hydrolysis, they are lost from the glands as NEFAs. This fits with the data of Enjalbert *et al.* (1998) and others where consideration of TAGs and NEFAs together leads to less variance than seen with either alone.

FABPs have been implicated in transmembrane and intracellular transport of fatty acids (Veerkamp *et al.*, 1991; Storch and Thumser, 2000). These are a group of tissue-specific proteins of about 14–15 kDa that bind long-chain fatty acids ( $C_{16}$ – $C_{20}$ ) with high affinity and a molar stoichiometry of 1:1. Most bind unsaturated fatty acids with higher affinity than saturated fatty acids. In addition to transport functions it has been proposed that they modulate specific enzymes of lipid metabolism, regulate expression of fatty acid-responsive genes, maintain cellular membrane fatty acid levels, and reduce the concentration of fatty acids in the cell, thereby removing their inhibitory effect on metabolic processes.

More recently, a greater role in regulating concentrations and transport of fatty acids in the cytosol has been proposed for the acyl-CoA-binding proteins (ACBP; Knudsen *et al.*, 2000). ACBP is an 86–103 residue protein, with a highly-conserved amino acid sequence, that binds long-chain acyl-CoA with a 10-fold higher affinity than does FABP (Rasmussen *et al.*, 1990), thus being much more effective in protecting membranes from damaging effects



**Figure 2.3.** Regulation of cytosolic free acyl-CoA concentration. The major factors affecting the cytosolic free long-chain acyl-CoA ester (LCA) concentration are the rates of LCA synthesis, uptake and consumption, the concentration of acyl-CoA-binding protein (ACBP), fatty acid-binding protein (FABP) and the acyl-CoA hydrolase activity. The two binding proteins buffer large fluctuations in free LCA concentration. The acyl-CoA hydrolases are suggested to function as a “scavenger” system to prevent the accumulation of free unprotected LCA and to ensure sufficient free CoA to support  $\beta$ -oxidation and other CoA-dependent enzymes. Sterol carrier protein 2 (SCP2), which binds LCA and very long chain-LCA, is localized in the peroxisomes. It is suggested that SCP2 acts as a peroxisomal pool former for LCA destined for  $\beta$ -oxidation in this organelle. (Adapted from Knudsen *et al.*, *J. Nutrition* 130:294S-298S, 2000, with permission).

of long-chain acyl-CoA esters. Many factors regulate the concentration of unbound acyl-CoA in cells (Figure 2.3). Under conditions in which the concentration of ACBP is not adequate to bind long-chain acyl-CoA, FABP takes over the buffering function, thus protecting cellular membranes from damage (Knudsen *et al.*, 2000). The concentration of ACBP is highest in liver cytosol, and interestingly, was found to be lowest in the mammary glands of the cow (Mikkelsen and Knudsen, 1987). One might expect, based on the presumption of an association of ACBP concentration with tissue lipid metabolic rates, that the concentration of ACBP might be high in mammary tissue.

### 2.2.5. Summary of the Supply of Long-Chain Fatty Acids to the Mammary Gland

More than 95% of  $C_{18}$  and longer-chain fatty acids in milk fat are derived from the blood TAG-rich lipoproteins. Non-esterified fatty acids are

taken up also, but net uptake is measurable only when the concentrations of NEFAs are high, notably in the early weeks of lactation when energy balance is negative and extensive mobilization of body lipid stores occurs. When cows are not in negative energy balance, adipose tissues contribute less than 15% of long-chain fatty acid uptake. Three-fourths of intestinal lipoprotein TAGs are taken up by lactating mammary glands. The proportion of the palmitic acid in milk fat derived from blood lipids is variable. When dietary fat intake is low, nearly all of palmitic acid is synthesized *de novo* in mammary tissue. As fatty acid uptake from blood TAGs increases, the proportion of palmitic acid from *de novo* synthesis decreases to 30% or less of the total.

### 2.3. Uptake of Non-Lipid Metabolites by Lactating Mammary Glands

In addition to long-chain fatty acids from plasma, the major nutrients utilized for milk fat synthesis are glucose, acetate and  $\beta$ -hydroxybutyrate. Kinetics for the uptake of these from blood were reported by Miller *et al.* (1991). Glucose is absolutely required for milk synthesis, being a precursor for lactose or other carbohydrates, or both, in all terrestrial mammals (Oftedal and Iverson, 1995).

In fasting animals, the arterial supply of glucose is limiting for milk synthesis (Chaiyabutr *et al.*, 1980), whereas in fed animals glucose uptake is independent of arterial concentration (Miller *et al.*, 1991). In the lactating mammary tissues of both ruminants and non-ruminants, glucose is taken up *via* facilitative transport systems, namely, the insulin-independent GLUT1 and probably a  $\text{Na}^+$ -dependent glucose transporter (Zhao *et al.*, 1996; Shennan and Peaker, 2000; Nielsen *et al.*, 2001). There is no evidence for the insulin-sensitive GLUT 4 transporter in lactating mammary tissues of either rats or cattle. Baldwin *et al.* (1980) suggested, on the basis of the limited arterio-venous difference data available in the literature at that time, that Michaelis-Menten type equations could be used to describe glucose uptake across the bovine udder. The data they collected were from a number of different studies and were highly scattered, such that the statistical fit to the data was improved only slightly by using a Michaelis-Menten as compared to a linear equation. Uptake of  $\beta$ -hydroxybutyrate and TAGs by the lactating mammary glands were well described by Michaelis-Menten relationships, whereas the uptake of acetate was strongly linear (Baldwin *et al.*, 1980). These authors cautioned that the uptake of metabolites by lactating mammary glands are interrelated, such that mathematical descriptions for individual nutrients are not unique, but are influenced by the

availability and uptake of other nutrients. In summarizing an extensive arterio-venous difference study on nutrient uptakes by cows' udders, these workers re-emphasized this point and concluded that factors other than arterial blood glucose concentration govern glucose uptake and utilization (Miller *et al.*, 1991; Baldwin, 1995).

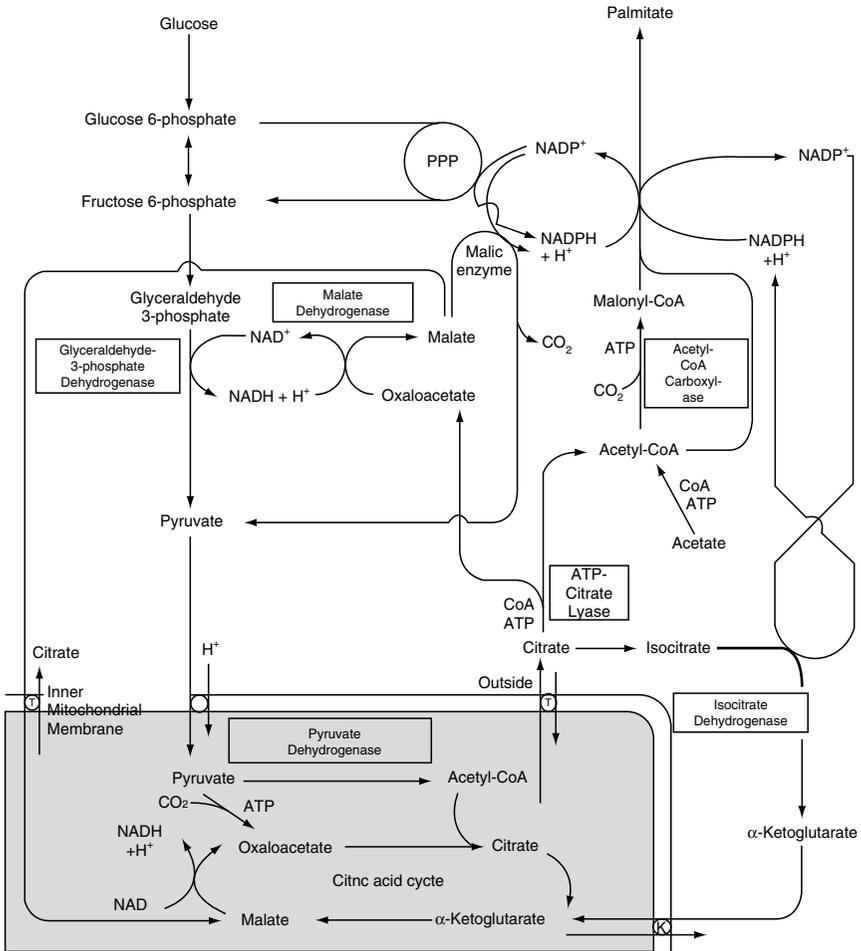
A serine/threonine protein kinase, Akt, is thought to regulate the expression or function of glucose transport proteins in adipose tissue, and also may represent a central signalling molecule in mammary gland development and function. Expression of constitutively activated Akt in mammary glands of transgenic mice resulted in precocious lipid accumulation; fat content of the milk was 65 to 70% by volume, compared with 25 to 30% in wild-type mice (Schwertferger *et al.*, 2003).

## 2.4. Fatty Acid Synthesis in Mammary Glands

Fatty acid synthesis in lactating mammary glands was discussed in detail by Hillgartner *et al.* (1995) and Barber *et al.* (1997). Comparative aspects of fatty acid synthesis in mammary glands of ruminants and non-ruminants were reviewed by Dils (1986) and an in-depth review of lipid metabolism in ruminant mammary glands was provided by Moore and Christie (1981). Requirements for fatty acid synthesis are a carbon source and reducing equivalents in the form of NADPH + H<sup>+</sup>. In ruminants, acetate and  $\beta$ -hydroxybutyrate are the primary carbon sources utilized, while glucose and acetate are the primary sources of reducing equivalents (Bauman and Davis, 1974). In non-ruminants, glucose is the primary source of both carbon and reducing equivalents (Figure 2.4). Enzyme activities and utilization of substrates by mammary tissues are increased dramatically relative to those of non-mammary tissues at the onset of lactation (Bauman and Currie, 1980; Vernon *et al.*, 1987).

### 2.4.1. Sources of Carbon and Reducing Equivalents for Fatty Acid Synthesis

The basic starting substrate for fatty acid synthesis is acetyl-CoA (see below). In ruminants, the provision of this substrate is straightforward. Acetate from blood (+ CoA + ATP) is converted by the cytosolic acetyl-CoA synthase (EC 2.3.1.169) to AMP and acetyl-CoA, which can then be used for fatty acid synthesis. In non-ruminants, glucose is converted *via* the glycolytic pathway to pyruvate, which is, in turn, converted to acetyl-CoA in mitochondria. Acetyl-CoA thus formed is converted to citrate which passes out to the cytosol where it is cleaved by ATP-citrate lyase (EC 2.3.3.8) to acetyl-CoA + oxalacetate (OAA). This "transport" of acetyl-CoA from



**Figure 2.4.** The provision of acetyl-CoA and NADPH for lipogenesis. PPP, pentose phosphate pathway; T, tricarboxylate transporter; K, α-ketoglutarate transporter. In ruminants, pyruvate dehydrogenase, ATP-citrate lyase and malic enzyme activities are low and perhaps non-functional. (From Murray *et al.*, 1988. *Harper's Biochemistry*, 21st edn, p. 207, Appleton and Lange, Norwalk, CT; reproduced with permission of The McGraw-Hill Companies).

mitochondria to the cytosol also results in the transport of OAA to the cytosol. OAA cannot move freely across the mitochondrial membrane as can pyruvate and citrate, but it can be reduced by  $NADH + H^+$  to form malate, which is converted by the malic enzyme (EC 1.1.1.40) to pyruvate and  $NADPH + H^+$ . Pyruvate can enter the mitochondrion and be converted to

OAA by pyruvate carboxylase (EC 6.4.1.1), thereby completing the net transport of the C<sub>2</sub> unit (acetate) from the mitochondrion to the cytosol with the added advantage of having converted a reducing equivalent as NADH + H<sup>+</sup> to NADPH + H<sup>+</sup>. This mechanism of "C<sub>2</sub> transport" provides up to 50% of the NADPH + H<sup>+</sup> for fatty acid synthesis in non-ruminants.

The reductive steps of FAS have a specific requirement for NADPH, whereas glycolysis, also a cytosolic process, generates NADH. In non-ruminants, some of the required NADPH is generated in the first two oxidative steps in the pentose phosphate pathway by glucose-6-phosphate dehydrogenase and 6-phospho-gluconate dehydrogenase, respectively. This provides perhaps one-half of the reducing equivalents required for fatty acid synthesis. In rats and mice, the remainder is generated as an adjunct to the mechanism of C<sub>2</sub> transport from the mitochondrion to the cytosol discussed above (Figure 2.4). The activities of both citrate lyase and malic enzyme increase with high carbohydrate diets in non-ruminants. The activities of these latter enzymes are low in ruminants, sows (Bauman *et al.*, 1970) guinea pigs, and rabbits (Crabtree *et al.*, 1981), probably reflecting the greater availability of acetate as a lipogenic precursor in these species or the absence of the need to transport C<sub>2</sub> units from the mitochondrion to the cytosol, or both.

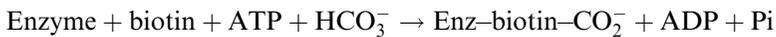
Glucose oxidation *via* the pentose phosphate pathway (PPP) is an equally important source of NADPH + H<sup>+</sup> in ruminants and also provides glycerol-3-phosphate for fatty acid esterification as an alternative to the glycolytic pathway. Glucose oxidation *via* the PPP accounts for 10 to 40% of ruminant mammary glucose utilization and 25 to 40% of mammary CO<sub>2</sub> output (Bickerstaffe *et al.*, 1974; Cant *et al.*, 1993). Wood *et al.* (1965) examined glucose utilization in perfused cow udders. Twenty-three to 30% of glucose was metabolized *via* the PPP, 10% was utilized in the Emden-Meyerhof pathway, and 60 to 70% was converted to lactose. A similar proportion was oxidized in the PPP in rats, but only 10 to 20% was utilized for lactose and up to 50% underwent glycolysis to provide C<sub>2</sub> units for lipogenesis (Katz and Wals, 1972).

ATP-citrate lyase activity is low in ruminants (Ballard *et al.*, 1969), and originally was considered to be the limiting factor that blocks the incorporation of glucose carbon into fatty acid carbon in ruminants. However, physiologically, this is not the reason. The negative feedback of acetyl-CoA formed from acetate on pyruvate dehydrogenase probably results in essentially complete inhibition of the enzyme activity in mammary as well as other tissues, thereby yielding a net sparing of glucose which is appropriate to the limited availability of glucose in these species. Inhibition of pyruvate dehydrogenase by acetyl CoA *in vivo* is consistent with the low incorporation of

pyruvate and lactate into fatty acids *in vivo* and also the incorporation of significant amounts of lactate and pyruvate carbon into fatty acids by bovine mammary tissue at high substrate concentrations *in vitro* in the absence of acetate (Forsberg *et al.*, 1985; Torok *et al.*, 1986). Conversion of glucose to fatty acids is low even in the absence of acetate, indicating a block somewhere between fructose-6-phosphate and pyruvate, possibly at phosphofructokinase (PFK) or in triose-phosphate metabolism, but this issue has not received any significant attention. Acetyl-CoA is a strong positive effector of pyruvate carboxylase. Citrate occurs at a rather high concentration in milk, being generated in the citric acid cycle from acetate, and the carboxylation of pyruvate and propionate. Because acetyl-CoA transport from mitochondria to the cytosol is not necessary in ruminants, cytosolic OAA and malate are not generated, so that  $\text{NADPH} + \text{H}^+$  from the malic enzyme is not available to support fatty acid synthesis. Instead, citrate diffuses from the mitochondrion to the cytosol and is converted to isocitrate, which is used to generate the  $\text{NADPH} + \text{H}^+$  required *via* the cytosolic NADP-isocitrate dehydrogenase (EC 1.1.1.42), accounting for as much as 50–60% of total NADPH used by ruminant fatty acid synthase (Mellenberger *et al.*, 1973; Mellenberger and Bauman, 1974). The product of this reaction,  $\alpha$ -ketoglutarate, is transported back into the mitochondrion (Figure 2.4). A unique aspect of this pathway is that NADPH is generated by oxidation of acetate, thus sparing glucose (Bauman *et al.*, 1970). High-fat diets reduce *de novo* fatty acid synthesis, thus decreasing the demand for reducing equivalents. This spares mammary glucose, freeing it for lactose synthesis (Cant *et al.*, 1993). Isocitrate oxidation is decreased, causing citrate concentration in milk to increase (Faulkner and Pollock, 1989).

### 2.4.2. Acetyl-CoA Carboxylase

The first committed step for the incorporation of acetate carbon into fatty acids is mediated by acetyl-CoA carboxylase (ACC; EC 6.4.1.2) in two steps, as follows (Allred and Reilly, 1997):



Five transcripts of the gene for ACC $\alpha$  have been described (Kim, 1997). These occur by two independent promoters, PI and PII, and differential splicing of the primary transcripts. Transcripts derived from PI have been characterized in adipose tissue while those from PII are found in mammary tissue (Kim, 1997). A third promoter (PIII) has been characterized in ovine mammary glands; it generates a transcript encoding an enzyme with an alternative N-terminus. Whereas PI was strongly expressed in bovine

udder (Mao *et al.*, 2001), it was not detected in the udder of lactating sheep by Travers *et al.* (2001). Both PII and PIII transcripts increased with lactation in sheep (Clegg *et al.*, 2001); PII was strongly induced (~10-fold) by lactation in rats (Ponce-Casteñeda *et al.*, 1991), but increased only about 3-fold in cows (Mao and Seyfert, 2002). The PIII promoter is strongly induced by lactation, being stimulated by prolactin *via* STAT 5 activation (Mao *et al.*, 2002). Molenaar *et al.* (2003) reevaluated all three promoters in mammary epithelial cells from cows and sheep, showing that all were active in both species. However, whereas all three promoters were shown to be relevant for milk fat synthesis in cattle, only PII and PIII were crucial for milk fat synthesis in sheep.

In liver, adipose, and possibly mammary, tissues, ACC is regulated acutely by phosphorylation/dephosphorylation and by allosteric mechanisms involving fatty acids (fatty acyl-CoA) and citrate, whereas the amount of protein is regulated by several hormones, including insulin, growth hormone, possibly *via* IGF-1, and prolactin (Kim *et al.*, 1992; Allred and Reilly, 1997; Barber *et al.*, 1997). Short-term regulation of ACC in liver and adipose tissues is well characterized. Very few studies of ruminant mammary tissue, where some of the same mechanisms may be present, have been reported. A possible reason for this deficiency is that the study of acute hormonal actions, which involve phosphorylation and dephosphorylation, require the use of cells with intact membranes. The high level of connective tissue in ruminant mammary tissue requires extensive incubation with collagenase to permit the isolation of secretory cells and these, most often, have severely damaged cell membranes, which are then unable to respond to hormonal signals.

Short-term hormonal regulation of ACC is achieved by covalent modifications of the enzyme by phosphorylation or dephosphorylation, which either increase or decrease its activity. These changes in enzyme activity are observed within minutes of exposure to hormones and thus are not likely due to changes in the amount of enzyme (Kim, 1983). Lee and Kim (1979) reported that incubation of rat adipocytes with epinephrine doubled the incorporation of <sup>32</sup>P into ACC within 30 minutes and reduced enzyme activity by 61%. Witters *et al.* (1979) established a similar relationship between phosphorylation and inactivation of rat hepatocyte ACC following glucagon treatment.

Rat liver ACC which has been phosphorylated and inactivated by a cyclic AMP—dependent protein kinase can be dephosphorylated and reactivated by incubation with a protein phosphatase (Curtis *et al.*, 1973). Similarly, ACC purified from rat or rabbit mammary glands are activated by dephosphorylation on incubation with a protein phosphatase (Hardie and Cohen, 1979; Hardie and Guy, 1980).

An early concern in studies of the activation of ACC by citrate was that the concentration of citrate required for activation was higher than that normally observed in tissues. Thus, it was of interest to find that the  $K_m$  for the binding of citrate to phosphorylated ACC of 2.4 mM was reduced to 0.2 mM by dephosphorylation (Carlson and Kim, 1974). Further, the dephosphorylated form of ACC is more susceptible to inhibition by palmitoyl-CoA. Also, phosphorylation and inactivation of ACC is accompanied by conversion of active polymers to inactive protomers (Shiao *et al.*, 1981). Mabrouk *et al.* (1990) found that inactive protomers isolated from the livers of rats injected with glucagon have a higher phosphate content than protomers isolated from the livers of control animals.

The signal transduction system responsible for ACC phosphorylation and inactivation has been studied in considerable detail. ACC isolated from rat mammary glands or rat livers is phosphorylated and inactivated by cAMP-dependent kinases. The  $^{32}\text{P}$ -labeled peptide isolated from rat adipocyte ACC after treatment with epinephrine and tryptic digestion co-migrated with the  $^{32}\text{P}$ -labeled peptide isolated after phosphorylation of purified ACC by cAMP-dependent kinase. There are three cAMP-independent protein kinases. Of these, ACC kinase-3 is stimulated by phosphorylation and AMP and is referred to as AMP-activated protein kinase. While the cAMP-dependent protein kinase phosphorylates Ser<sub>77</sub> and Ser<sub>1200</sub> and causes moderate inactivation of ACC, the AMP-activated protein kinase phosphorylates at Ser<sub>79</sub> and Ser<sub>1200</sub> and causes a dramatic inactivation of ACC purified from lactating rat mammary glands (Munday *et al.*, 1988). Further studies (Haystead *et al.*, 1990) established that hormone-induced phosphorylation of Ser<sub>77</sub> by cAMP-dependent protein kinases may not occur *in vivo*, but rather, that the cAMP-elevating hormones activate the AMP-activated protein kinase *via* a kinase-kinase system and that the phosphorylation at Ser<sub>79</sub> by this enzyme, in turn, is the mechanism of inactivation of ACC.

In contrast to the acute inactivation of ACC by cAMP-elevating hormones, it is well established that insulin stimulates fatty acid synthesis in several tissues, including lactating rat mammary acini, within a few minutes of treatment (Williamson *et al.*, 1983). Responses of ruminant lipogenic tissues to insulin are generally far less dramatic than in rodents. For example, Yang and Baldwin (1973) found only a doubling of fatty acid synthesis in bovine adipocytes treated with insulin during a 3-hour incubation. Lactating cow mammary tissue does not appear to exhibit acute responses to insulin *in vitro* (Forsberg *et al.*, 1985; Laarveld *et al.*, 1985) although the tissue does have insulin receptors (Oscar *et al.*, 1986).

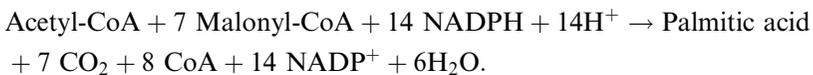
The insulin-induced enhancement of fatty acid synthesis in non-ruminant adipose and liver tissues is associated with an increase in the

phosphorylation of ACC. Analyses of tryptic peptides derived from  $^{32}\text{P}$ -labeled ACC from insulin-treated lipogenic tissues indicated that the peptides labelled were clearly separate and distinct from those induced by epinephrine. The peptide labelled by insulin treatment is referred to as the “I-peptide” (Brownsey and Denton, 1982). The polymerized form of ACC, which is most active, was found to have increased phosphorylation of the “I-peptide” (Borthwick *et al.*, 1987). These workers (Borthwick *et al.*, 1990) established that the increases in phosphorylation and activity of ACC occurred in parallel, leading to the development of the concept that hormones such as epinephrine, acting *via* cAMP, inactivate ACC by phosphorylating sites separate from those phosphorylated by insulin, which activate ACC.

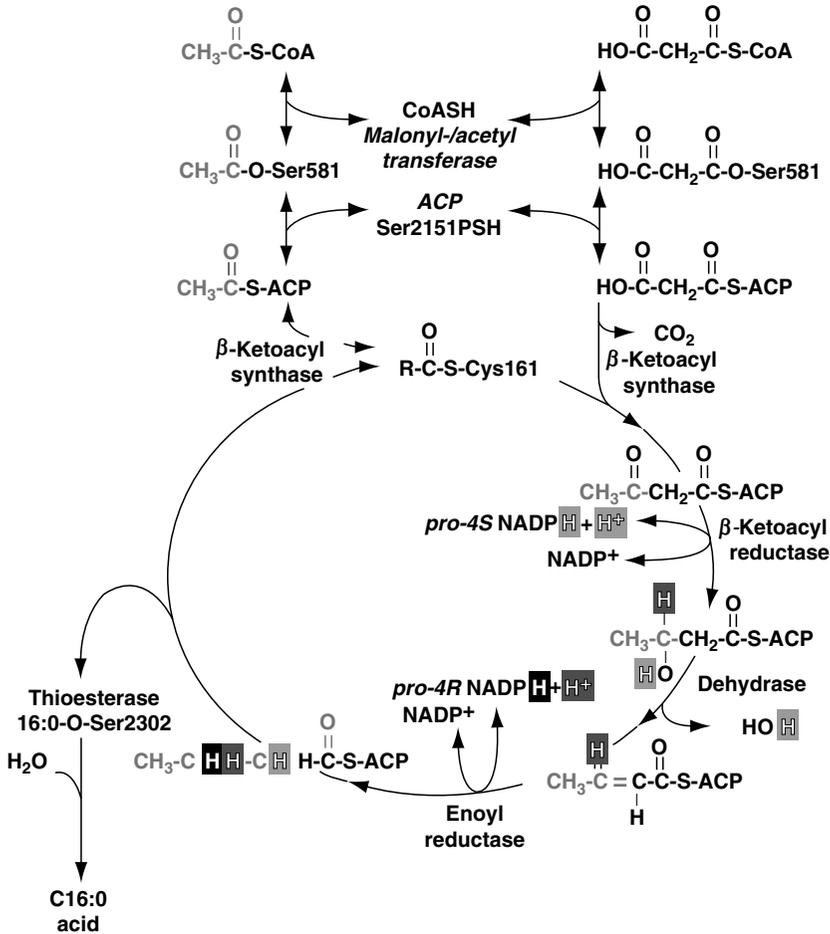
It has been reported also that epidermal growth factor stimulates fatty acid synthesis and the phosphorylation of ACC in rat liver and adipose tissues (Holland and Hardie, 1985) through phosphorylation of the “I-peptide,” suggesting that several peptide hormones sharing homology with insulin, such as IGF-1, could enhance lipogenesis similarly.

### 2.4.3. Fatty Acid Synthase

The animal fatty acid synthase (FAS; EC 2.3.1.85) is one of the most complex multifunctional enzymes that have been characterized, as this single polypeptide contains all the catalytic components required for a series of 37 sequential transactions (Smith, 1994). The animal FAS consists of two identical polypeptides of approximately 2500 amino acid residues (MW, *ca.* 270 kDa), each containing seven catalytic subunits: (1) ketoacylsynthase, (2) malonyl/acetyl transferase, (3) dehydrase, (4) enoyl reductase, (5)  $\beta$ -keto reductase, (6) acyl carrier protein (ACP), and (7) thioesterase. Although some components of the complex are able to carry out their respective catalytic steps in the monomeric form, only in the FAS dimer do the subunits attain conformations that facilitate coupling of the individual reactions of fatty acid synthesis to occur (Smith *et al.*, 2003).



The entire sequence is described in exquisite detail by Smith *et al.* (2003; see Figure 2.5). The first step is the sequential transfer of the primer, usually acetyl-CoA, to the serine residue of the acyl transferase, then to the ACP, and finally to  $\beta$ -ketoacyl synthase. The chain extender substrate, usually malonyl-CoA, is transferred *via* the serine residue of the acyl transferase to ACP. Condensation is accomplished by  $\beta$ -ketoacylsynthase, aided by the energetically-favourable decarboxylation of the malonyl residue,



**Figure 2.5.** Reaction sequence for the biosynthesis of fatty acids *de novo* by the animal FAS. The condensation reaction proceeds with stereochemical inversion of the malonyl C-2, the  $\beta$ -ketoacyl moiety is reduced by NADPH to D- $\beta$  hydroxyacyl moiety, which then is dehydrated to a *trans*-enoyl moiety; finally, the enoyl moiety is reduced to a saturated acyl moiety by NADPH, with the simultaneous addition of a solvent proton. The two C atoms at the methyl end of the fatty acid are derived from acetyl-CoA, the remainder from malonyl-CoA. The entire series of reactions takes approximately 1 second. PSH, phosphopantetheine. (Reprinted from Prog. in Lipid Res., vol. 42, S. Smith, A. Witkowski and A.K. Joshi, Structural and functional organization of the animal fatty acid synthase, pp. 289–317, copyright (2003), with permission from Elsevier).

resulting in the release of  $\text{CO}_2$ . Note that the acetyl moiety originally bound to ACP to initiate the synthetic sequence becomes the methyl-terminal of the growing acyl chain. Sequential reactions catalyzed by  $\beta$ -ketoacyl reductase,

dehydrase and enoyl reductase result in the formation of butyryl-*S*-ACP, which re-enters the cycle through condensation with another malonyl-ACP. Cycling continues, with the addition of C<sub>2</sub> units from malonyl-CoA until the chain length of the nascent fatty acid reaches C<sub>12</sub> to C<sub>16</sub>, when a thioesterase specific for that chain length releases the fatty acid and terminates the cycle.

#### 2.4.4. Regulation of Acyl Chain Length

The chain length of the fatty acid product is influenced by numerous factors. The same acyl transferase accomplishes loading of both the acetyl and malonyl substrates. The process is random. Both substrates are exchanged rapidly between CoA and ACP until a combination occurs that permits chain initiation or a continuation of elongation (Smith *et al.*, 2003). The catalytic rate of the acyl transferase increases as chain length progresses from C<sub>2</sub> to C<sub>12</sub>; the total concentration of covalently-bound saturated intermediates decreases with increasing chain lengths up to C<sub>14</sub>, implying that the early  $\beta$ -ketoacylsynthase reactions are slower than the later ones (Smith *et al.*, 2003). Mass action coefficients for chains longer than C<sub>12</sub> become progressively smaller, with little accumulation of C<sub>18</sub>. Acyltransferase is not entirely specific for the acetyl and malonyl moieties.  $\beta$ -Ketobutyryl,  $\beta$ -hydroxybutyryl and crotonyl residues all are incorporated (Dodds *et al.*, 1981; Joshi *et al.*, 1997). Indeed, Kumar and associates (Nandedkar *et al.*, 1969; Lin and Kumar, 1972) have shown that lactating mammary glands utilize butyryl-CoA more efficiently than acetyl-CoA as a “primer” for FAS. Further, these authors showed that butyryl-CoA is synthesized from acetyl-CoA by, essentially, a reversal of  $\beta$ -oxidation in both the liver and mammary glands of rabbits, rats and cows. Knudsen and Grunnet (1980) showed that butyryl-CoA is superior to acetyl-CoA as a primer for FAS, but only in ruminants. The acyl-CoA dehydrogenase associated with  $\beta$ -oxidation is an FAD-linked dehydrogenase, which renders  $\beta$ -oxidation essentially irreversible on thermodynamic grounds. The enzyme that catalyzes the reverse reaction in the Kumar scheme is linked *via* NADPH + H, which renders the reduction of crotonyl-CoA thermodynamically favourable. Thus, this enzyme, unique to the reversal of  $\beta$ -oxidation, could be called “crotonyl-CoA reductase.” This activity was much lower in rat adipose and pigeon liver tissues. This synthetic pathway is independent of malonyl-CoA and thus is not subject to regulation by acetyl-CoA carboxylase. These observations explain the incorporation of  $\beta$ -OH butyrate as the methyl terminal C<sub>4</sub> moiety of up to 50–60% of fatty acids synthesized *de novo* by lactating mammary glands (Palmquist *et al.*, 1969; Smith *et al.*, 1974). Also, small quantities of C<sub>6</sub>, C<sub>8</sub> and C<sub>10</sub> in milk fat may be synthesized *via* this pathway. Propionic acid is incorporated as a primer leading to the synthesis of an odd

number of carbon atoms in the acyl chain. The branched-chain volatile acids, *iso*-valeric, *iso*-butyric and 2-methyl butyric, also can serve as primers, giving rise to *iso*- and *ante*-*iso* acyl chains (Massart-Leen *et al.*, 1981; Ha and Lindsay, 1990). Methyl-malonyl-CoA also may be incorporated, substituting for malonyl-CoA, leading to multi-branched acyl products; synthesis of these is terminated with a chain length less than 16 carbons, and the rate of synthesis is one-tenth that for straight-chain products (Smith, 1994). Some branched-chain products, in particular 13-methyl tetradecanoic acid, have been shown to have regulatory effects on cell function (Yang *et al.*, 2000; Parodi, 2003).

Because the acyl transferase releases intermediates, some of the intermediates may escape transfer to the  $\beta$ -ketoacylsynthase, resulting in short-chain fatty acids being incorporated into milk fat. In non-ruminants, this is limited mainly to butyrate, whereas the more relaxed specificity of the acyl transferase in ruminants allows medium-chain acyl-CoA to be released in significant amounts (Hansen and Knudsen, 1980). An increased concentration of malonyl-CoA in the medium increases the relative proportions of longer-chained fatty acids (Knudsen, 1979; Knudsen and Grunnet, 1982). Indeed, this can be inferred from studies in which intestinal glucose supply (Hurtaud *et al.* 2000) or systemic insulin concentrations (Griinari *et al.* 1997) were increased experimentally. Removal of intermediate-chain length acyl-CoA is dependent on the presence of an acceptor, such as albumin (Knudsen and Grunnet, 1982),  $\alpha$ -glycerol-phosphate or diacylglycerol. The incorporation of fatty acids into TAGs is greatly enhanced by the presence of non-limiting concentrations of  $\alpha$ -glycerolphosphate or diacylglycerol (Grunnet and Knudsen, 1981; Hansen *et al.*, 1984b).

Regulation of chain termination reactions in ruminant and non-ruminant mammary glands was discussed in detail by Smith (1994; see also Barber *et al.*, 1997). In non-ruminant mammary tissue, medium-chain fatty acids are generated by a novel chain-terminating enzyme that is not part of the FAS complex, known as thioesterase II to distinguish it from the chain-terminating enzyme (thioesterase I) associated with FAS (Smith, 1994). Thioesterase II, though a 29 kDa protein independent of FAS, appears to function identically to thioesterase I. However, its specificity differs, producing C<sub>8</sub> to C<sub>14</sub> fatty acids.

#### 2.4.4.1. Elongation of C<sub>16</sub> acyl chain

Bishop *et al.* (1969) reported that 4.6% of intravenously-infused methyl [<sup>3</sup>H] palmitate was incorporated into longer-chained FA of milk fat when a cow was fed a low-fat diet, whereas Palmquist *et al.* (1969) reported no significant labelling of C<sub>18</sub> acids in milk fat after intra-mammary infusions of

$1\text{-}^{14}\text{C}$ -acetate. Chain lengthening of long-chain CoA esters occurs in the microsomes, using malonyl-CoA as the acetyl donor (Bernert and Sprecher, 1979). This is not a significant pathway for the supply of  $\text{C}_{18}$  fatty acids for milk fat synthesis.

## 2.5. Stearoyl-CoA Desaturase

Stearoyl-CoA desaturase (SCD; EC 1.14.19.1) is the rate-limiting enzyme for the conversion of saturated to monounsaturated fatty acids. As such, it plays a major role in regulating the unsaturation of membranes and TAG composition, and evidence indicates that SCD plays an important regulatory role in lipid metabolism. SCD-1-deficient mice exhibit increased fatty acid oxidation and reduced lipid synthesis; a significant proportion of the metabolic effects of leptin may result from inhibition of this enzyme (Ntambi and Miyazaki, 2004). Its role is especially important in ruminants, in which the majority of absorbed  $\text{C}_{18}$  fatty acids are in the form of stearate as a result of ruminal biohydrogenation. The activity of SCD in ruminants is high in lactating mammary gland and adipose tissue, and somewhat lower in intestinal tissue. SCD activity is low in non-lactating mammary tissue, being induced by lactation (Kinsella, 1972); though normally inactive in ruminant liver, SCD may be induced in liver and muscle by high-fat diets (Chang *et al.*, 1992). SCD activity was not measurable in rat (Kinsella, 1970) or rabbit (Bickerstaffe and Annison, 1970) mammary tissue; possibly the mRNA is present but its translation is down-regulated by dietary unsaturated fat, as demonstrated in mice (Singh *et al.*, 2004). Whereas SCD is characterized by three isoforms in mice, only one gene is expressed in ruminants and humans (Ward *et al.*, 1998; Ntambi and Miyazaki, 2004). In ruminants, the SCD gene has been localized to bovine and caprine chromosome 26q21, and ovine chromosome 22q21 (Bernard *et al.*, 2001).

SCD is located in the endoplasmic reticulum; its primary substrates are stearoyl-CoA and palmitoyl-CoA, whereas considerably lower activity is observed with myristoyl-CoA as substrate (Bickerstaffe and Annison, 1970). Desaturation of a range of *trans* monoenes occurs also (Mahfouz *et al.*, 1980), yielding *trans*-x, *cis*-9 octadecadienoic acids as products. This is particularly important considering the large amount of *trans* octadecenoic acids that is formed in the rumen by biohydrogenation (Shingfield *et al.*, 2003); among these, the quantitatively most important is desaturation of vaccenic acid (*trans*-11  $\text{C}_{18:1}$ ) to rumenic acid (*cis*-9, *trans*-11  $\text{C}_{18:2}$ ), commonly called conjugated linoleic acid (CLA). Desaturation of *trans*-7  $\text{C}_{18:1}$  to yield *trans*-7, *cis*-9  $\text{C}_{18:2}$  has been reported also (Corl *et al.*, 2002).

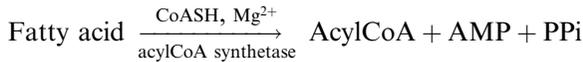
Considerable variation among cows (Kinsella, 1972; Kelsey *et al.*, 2003) and breeds (DePeters *et al.*, 1995) in the extent of stearic acid desaturation and in mRNA expression (Taniguchi *et al.*, 2004) has been observed. SCD mRNA expression is down-regulated by *trans*-10, *cis*-12 C<sub>18:2</sub> (Choi *et al.*, 2000), which is formed in the rumen in small amounts when milk fat-depressing diets are fed (Bauman and Griinari, 2003).

## 2.6. Triacylglycerol Synthesis

Enzymes for triacylglycerol synthesis are associated with the endoplasmic reticulum and the inner and outer mitochondrial membranes (Coleman *et al.*, 2000). Glycerol-3-phosphate required for esterification of fatty acids is generated by glycolysis or by phosphorylation of free glycerol by glycerol kinase, which is active both in ruminant and non-ruminant tissues (McBride and Korn, 1964; Kinsella, 1968; Bickerstaffe and Annison, 1971). Although some free glycerol may be taken up from blood during lipolysis of lipoprotein TAGs, most glycerol-3-phosphate is probably derived from glycolysis (Bickerstaffe and Annison, 1971).

Enzymes and regulation of TAG synthesis have been reviewed in depth by Coleman *et al.* (2000, 2004).

For metabolism to occur, fatty acids must be activated to their acyl CoA esters:



Because the pyrophosphate, PPi, is hydrolyzed rapidly to two inorganic phosphates, Pi, the reaction is strongly in the direction of acyl CoA formation and the energy requirement for activation is the equivalent of two high energy bonds. Acyl CoA synthetase is expressed in several isoforms and is distributed on the cytosolic surfaces of the endoplasmic reticulum, peroxisomal and outer mitochondrial membranes. Activity of this enzyme responds to changes in physiological state, suggesting that it may play a role in regulating the entry of fatty acids into synthetic or oxidative pathways (Coleman *et al.*, 2002).

Acylation of glycerol-3-phosphate is the first committed step in TAG synthesis and the activity of acyl CoA:glycerol-*sn*-3-phosphate acyl transferase (GPAT) is the lowest of the transacylation enzymes in this pathway, also suggesting a regulatory role in TAG synthesis (Coleman *et al.*, 2004). Although microsomal GPAT constitutes the majority of total GPAT activity in most tissues and can be activated and inhibited by phosphorylation and

dephosphorylation, its role in TAG synthesis is uncertain. It has been purified only partially (Coleman *et al.*, 2004). Mitochondrial GPAT, located on the mitochondrial outer membrane, is characterized more thoroughly. Its activity is influenced by nutritional and hormonal changes; transcription is enhanced during adipocyte differentiation, and it responds inversely to fasting and feeding and to phosphorylation and dephosphorylation (Coleman *et al.*, 2004). Cooper and Grigor (1980) reported that acylation at *sn*-1 in rat mammary TAGs favours oleic acid, whereas palmitic acid predominates at *sn*-2. This pattern is true in most species, although in the cow, palmitic acid is found nearly equally at the *sn*-1 and *sn*-2 positions (Christie, 1985; Parodi, 1982; Jensen and Newburg, 1995). The product, 1-acyl-lysophosphatidate, is acylated by acyl CoA:1-acylglycerol-*sn*-3-phosphate acyltransferase. The microsomal fraction from lactating cow mammary gland transfers acyl chains C<sub>8</sub> to C<sub>18</sub> but not C<sub>4</sub> or C<sub>6</sub> to the *sn*-2 position of 1-acyl-lysophosphatidate. The chain length specificity is C<sub>16</sub> > C<sub>14</sub> > C<sub>12</sub> > C<sub>10</sub> > C<sub>8</sub>, similar to the pattern found at the *sn*-2 position of bovine milk fat (Marshall and Knudsen, 1977).

Phosphatidic acid (1,2 diacylglycerol-*sn*-3-phosphate) occupies a central point in lipid biosynthesis. It can be converted to CDP-diacylglycerol, a precursor for the biosynthesis of acidic phospholipids, or desphosphorylated to produce diacylglycerol, the precursor of TAGs, phosphatidylserine, phosphatidylcholine and phosphatidyl ethanolamine (Coleman *et al.*, 2004). In TAG synthesis, desphosphorylation of phosphatidic acid is mediated by phosphatidic acid phosphatase-1, a Mg<sup>2+</sup>-requiring enzyme that is transferred from the cytosol to the endoplasmic reticulum in the presence of fatty acids or acyl-CoA. Activity in liver is stimulated by glucagon, glucocorticoids, cAMP and growth hormone, and inhibited by insulin (Coleman *et al.*, 2004).

Diacylglycerol acyltransferase (*sn*-1,2-diacylglycerol transacylase) esterifies both long-chain and short-chain fatty acids at the *sn*-3 position. Regulation of total activity has been reported in liver and adipose tissues, but little information is available for mammary glands. Because it is up-regulated with increasing abundance of fatty acids, most likely it is in a highly active state in lactating mammary tissue. Mice that lack both copies of the gene for diacylglycerol acyltransferase 1 (DGAT 1) are unable to secrete milk (Smith *et al.*, 2000). This gene was mapped to a region close to the quantitative trait locus on bovine chromosome 14 for variation in milk fat content (Grisart *et al.*, 2001). Sequencing revealed frequency shifts at several positions on the gene between groups of animals with high or low breeding values for milk fat content. The most likely candidate for lower milk fat content was substitution of alanine for lysine in the enzyme (Winter *et al.*, 2002). The wild type allele (DGAT1<sup>K</sup>) exceeds the DGAT1<sup>A</sup> allele by

+0.34 percentage units in fat and +0.08 percentage units in protein, whereas milk and protein yields are reduced (Grisart *et al.*, 2001). A genotyping study of 38 *Bos indicus* and *Bos taurus* breeds from five continents showed that most domesticated dairy breeds have predominantly the DGAT1<sup>A</sup> allele, whereas DGAT1<sup>K</sup> was the major allele (69%) in the Jersey breed (Kaupe *et al.*, 2004).

In nearly all species studied, oleic acid is the fatty acid preferably esterified at the *sn*-3 of TAGs; C<sub>18:3n-3</sub> predominates in the koala and horse. In ruminant species, butyric or caproic acid is esterified exclusively at *sn*-3 and slightly exceeds the molar percentage of oleic acid esterified at that position (Parodi, 1982). Lin *et al.* (1976) examined the acyl specificity for TAG synthesis in lactating rat mammary glands, particularly with respect to the unique positioning of short-chain and medium-chain length fatty acids at *sn*-3. Whereas the acyl transferases for *sn*-1 and -2 showed high specificity for long-chain fatty acids (C<sub>16</sub> and C<sub>18</sub>), no such specificity was observed for acylation of diacylglycerol. They concluded that a lack of acyl chain specificity for this position caused accumulation of shorter acyl chains at *sn*-3.

Knudsen and colleagues (Hansen and Knudsen, 1980; Marshall and Knudsen, 1980; Grunnet and Knudsen, 1981; Hansen *et al.*, 1984a,b) examined in detail TAG synthesis and the specific incorporation of the short-chain and medium-chain fatty acids into TAGs in goat mammary glands. The synthesis of medium-chain fatty acids is dependent on simultaneous removal of the acyl-CoA produced by FAS, whereas long-chain fatty acids are released as free acids by thioesterase I. As described above, long-chain fatty acids are esterified preferentially at positions *sn*-1 and -2; the ready supply of diacylglycerols allows short-chain and medium-chain fatty acids to be esterified rapidly at *sn*-3, facilitating their removal from FAS. These studies have shown the importance of the rate of activation of fatty acids in the mammary gland relative to the rate of *de novo* synthesis and the supply of  $\alpha$ -glycerol phosphate for milk fat synthesis. If the supply of exogenous fatty acids were low, the relative concentration of short-chain and medium-chain fatty acids could be increased, even though total synthesis (yield) was not increased. Conversely, with an increasing supply of exogenous long-chain fatty acids, *de novo* synthesis may be reduced because they compete for the diacylglycerol transferase. Limiting the supply of  $\alpha$ -glycerol phosphate similarly would limit diacylglycerol supply, also causing *de novo* synthesis to be reduced. These observations demonstrate also that regulation of the relative proportions of short-chain, medium-chain and long-chain fatty acids is much more complex than simply by regulation of ACC. Finally, these authors concluded that a simple explanation for the unique occurrence of short-chain and medium-chain fatty acids in milk fat could be that

$\alpha$ -glycerol phosphate is rate-limiting for TAG synthesis in all ruminant tissues except mammary glands (Hansen *et al.*, 1984b).

Interestingly, for maximum TAG synthesis, a preference was shown for palmitoyl-CoA as substrate for the initial acylation of glycerol-3-phosphate (Kinsella and Gross, 1973), apparently accelerating the rate of supply of substrate as acceptor for *de novo*-synthesized fatty acids, whereas oleic acid reduced total *de novo* synthesis, apparently by competing with butyryl CoA for the esterification of diacylglycerol (Hansen and Knudsen, 1987).

### 2.6.1 Fatty Acid Esterification by the Monoacylglycerol Pathway

Evidence for the esterification of fatty acids *via* the 2-monoacylglycerol pathway was shown for mammary glands of guinea pigs by McBride and Korn (1964) and of goats by Bickerstaffe and Annison (1971). However, even though this is an important pathway for the esterification of fatty acids in the intestine, later studies have established that this pathway is not functional in mammary tissue (Christie, 1985; Hansen *et al.*, 1986).

## 2.7. Synthesis of Complex Lipids

The complex lipids in milk fat are comprised of the phosphoglycerides, phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidylinositol and plasmalogens. Also, the non-glyceride phospholipid, sphingomyelin, occurs in important amounts (Jensen, 2002). Bitman and Wood (1990) described the distribution of phospholipid classes in bovine milk and their fatty acid composition. The phospholipids comprise about 1% and cholesterol 0.4–0.5% of the total milk fat. These occur almost completely in the milk fat globule membrane.

The fatty acid composition of phosphoglycerides and sphingolipids, and their incorporation into membranes with cholesterol, are coordinately regulated processes in order to maintain membrane integrity and function. Coordination is mediated by sterol regulatory genes and sterol receptor element-binding proteins (SREBPs; Ridgway *et al.*, 1999). Glycosphingolipids are highly enriched in the outer leaflet of the apical plasma membrane domain of polarized epithelial cells. They are a key component of membrane lipid rafts and thus are involved in exocytosis of milk components. Their metabolites act as second messengers in regulating the expression of cell receptors (Hoekstra *et al.*, 2003). Sphingolipids are enriched in the milk fat globule membrane, and as a food component have been implicated in cell regulation and anti-cancer activity (Vesper *et al.*, 1999).

### 2.7.1. Synthesis of Phospholipids

Synthesis of phosphatidylcholine and phosphatidylethanolamine begins with activation of choline (or ethanolamine) with ATP *via* choline kinase to yield phosphocholine (phosphoethanolamine) + ADP; the activated base is transferred *via* CTP and phosphocholine cytidyl transferase to form CDP-choline (CDP-ethanolamine) and PPI. The base is then transferred to the *sn*-3 of diacylglycerol *via* phosphocholine diacylglycerol transferase to yield phosphatidylcholine (phosphatidylethanolamine) + CMP. The cytidyl transferase is believed to be the rate-limiting or regulatory step in the pathway. Phosphatidylserine is formed by a direct transfer and substitution of serine for ethanolamine in phosphatidylethanolamine. Phosphatidyl serine can be decarboxylated to form phosphatidylethanolamine.

Phosphatidylcholine is preferentially synthesized in lactating mammary tissue (Kinsella, 1973), possibly regulated by the differential activities of choline kinase and ethanolamine kinase. Choline kinase has a lower  $K_M$  and a higher  $V_{max}$  with its substrate than does ethanolamine kinase. Also, choline kinase is inhibited slightly by ethanolamine, whereas choline is a potent competitive inhibitor of ethanolamine kinase. Thus, the intracellular concentration of choline probably regulates the synthesis of these two phosphoglycerides (Infante and Kinsella, 1976).

The *sn*-1 and *sn*-2 acyl groups of phosphoglycerides differ among phospholipid classes (Bitman and Wood, 1990) and from the patterns of TAGs. However, the CDP choline:diacylglyceride transferase has little specificity for the molecular species of fatty acids in the diacylglycerol moiety. Therefore, molecular remodelling *via* specific phospholipases, followed by reacylation *via* specific acyl CoA transferases, may account for the unique fatty acid profiles of the various phosphoglycerols. Kinsella and Infante (1974) showed that the acyl CoA:1-acyl-*sn*-glycerol-3-phosphoryl choline acyl transferase preferentially esterified oleic acid at the *sn*-2 position, and that the predominant molecular species of phosphatidylcholine in mammary cells is diunsaturated.

Synthesis of phosphatidylinositol follows a slightly different pathway from the other phosphoglycerides. Phosphatidate is activated by CTP-phosphatidate cytidyl transferase to form CDP-diacylglycerol. Free inositol is then incorporated by CDP-diacylglycerol inositol transferase, with the release of CMP. Phosphatidylinositol usually constitutes less than 5% of total milk phospholipids (Bitman and Wood, 1990).

The glycerol ethers and plasmalogens are a unique class of phosphoglycerides that contain an ether-linked chain at the *sn*-1 position. These are formed by the incorporation of acyl CoA at the *sn*-1 position of dihydroxyacetone phosphate. The acyl group is then exchanged for a long-chain

alcohol to yield 1-alkyl dihydroxyacetone phosphate, which is reduced to 1-alkylglycerophosphate. This product is acylated to 1-alkyl, 2-acylglycerophosphate which then may be incorporated into various phosphoglycerols or acylated to form a neutral alkylglyceride. The alkyl residue of the phosphatidylethanolamine typically is desaturated at the 1,2 position, involving O<sub>2</sub> and NADPH to yield 1-alkenyl, 2-acyl-glycerol- 3-phosphatidylethanolamine (plasmalogen), found at a high concentration in the mitochondrial membrane. The alkylglycerols occur at very low concentrations in milk fat (~0.01% of the fat) but are 5- to 20-fold higher in the fat of colostrum (Ahrné *et al.*, 1980).

### 2.7.2. Sphingolipids

The sphingolipids are very complex molecules, occurring in numerous molecular forms (Jensen, 2002). The basic structure, sphingomyelin, consists of a complex unsaturated amino alcohol (sphingosine) ether-linked to phosphocholine and to an acyl chain by an amide bond. It contains no glycerol (Vesper *et al.*, 1999). In sphingomyelin synthesis, sphingosine is acylated by acyl-CoA to form a ceramide; phosphocholine is then transferred to a ceramide from phosphatidylcholine or from CDP-choline to yield sphingomyelin. Sphingomyelin constitutes about 25% of the total phospholipids in dairy products, which are the most abundant source of sphingolipids in the human diet. The sphingolipids are highly bioactive, and as such, are considered to be functional in foods (Vesper *et al.*, 1999; see Chapter 13). The neutral sphingolipids contain no phosphocholine, and therefore are not phospholipids. These are formed by addition of various sugar residues to ceramide. These can be quite complex (Jensen, 2002). Sphingolipids often contain very long-chain (C<sub>24</sub>) fatty acids, especially those found in the brain.

### 2.7.3 Cholesterol

Cholesterol makes up 95% of the total milk sterols and, because it is associated with the milk fat globule membrane, its content is highly correlated with the total fat content (Jensen, 2002). Only about 10% of the cholesterol in milk is esterified.

Cholesterol is taken up rapidly from plasma lipoproteins, and is synthesized also by the mammary glands (Clarenburg and Chaikoff, 1966). Quantitative contributions of each, and whether all lipoproteins or only chylomicra and VLDL contribute cholesterol, are less certain (Clarenburg and Chaikoff, 1966; Raphael *et al.*, 1975a,b). From steady-state estimates of labelled cholesterol in lipoproteins, plasma contributed 83% of milk cholesterol in lactating rats (Clarenburg and Chaikoff, 1966) and 45–50% in a lactating goat (Raphael *et al.*, 1975b). The mechanism of cholesterol uptake

also is not defined; however, an active cholesterol esterase is present in mammary tissue (Ross and Rowe, 1984; Shand and West, 1991; Small *et al.*, 1991), suggesting that both free and esterified lipoprotein cholesterol may be taken up and utilized.

## 2.8. Physiological Factors That Influence Milk Fat Composition

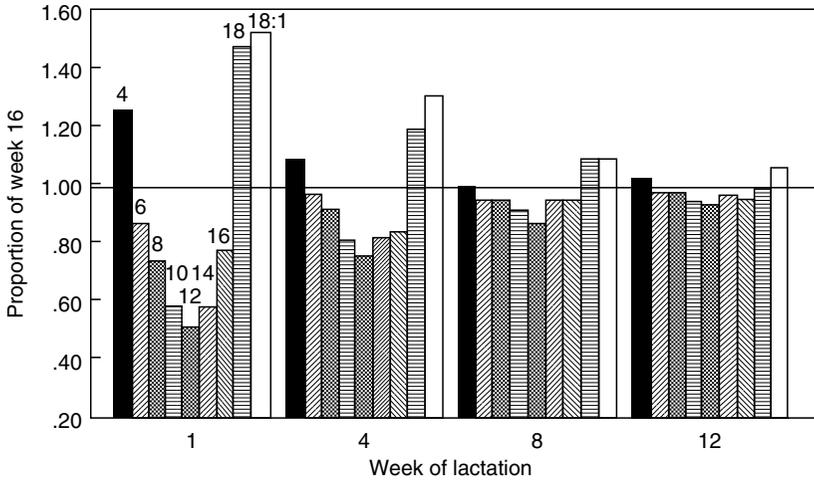
### 2.8.1. Genetics

In addition to the well-characterized differences among and within breeds of dairy cattle in milk fat content, differences occur also in fatty acid composition. Karijord *et al.* (1982) reported a positive genetic correlation between the proportions of short-chain and medium-chain ( $C_6$ – $C_{16}$ ) fatty acids and the amount of milk fat, whereas the correlation was negative for proportions of all  $C_{18}$  acids. They noted an especially high negative relationship between milk fat content and the proportion of  $C_{18:1}$ ; however, they did not separate the isomers of  $C_{18:1}$  and thus it is possible that the effect was caused by the presence of *trans*  $C_{18:1}$  isomers when  $C_{18:1}$  was in higher amounts (Bauman and Griinari, 2001). The positive correlation observed between short-chain fatty acids and milk fat level is consistent with data of Stull and Brown (1964) and Beaulieu and Palmquist (1995). Numerous studies indicate that SCD activity, and thus 18:1/18:0 ratio, varies among breeds (Beaulieu and Palmquist, 1995; DePeters *et al.*, 1995) and within a breed (Kelsey *et al.*, 2003).

### 2.8.2. Stage of Lactation

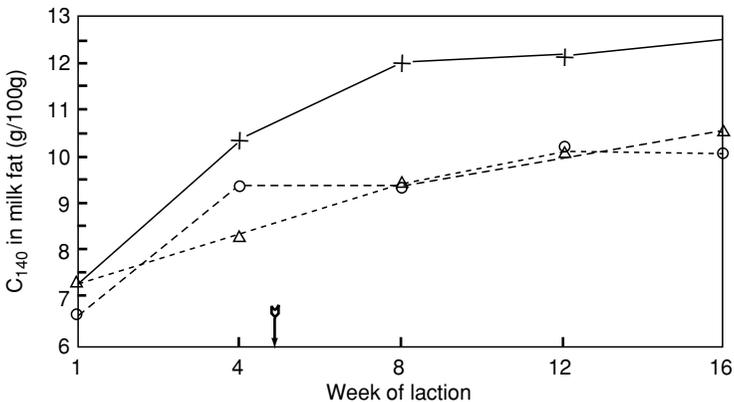
At parturition, the proportions of  $C_{12}$  to  $C_{16}$  fatty acids are relatively high in bovine colostrum. Proportions of short-chain fatty acids and stearic and oleic acids increase rapidly as the mobilization of adipose tissue commences, becoming relatively stable by one week after parturition (Laakso *et al.*, 1996).

Proportions and yields of fatty acids synthesized *de novo* in the mammary gland ( $C_6$  to  $C_{16}$ ) increase during the early weeks of lactation, with compensating decreases in the proportions of all  $C_{18}$  fatty acids (Figure 2.6; Karijord *et al.*, 1982; Lynch *et al.*, 1992). The proportion of butyric acid is high at parturition and does not increase with advancing lactation (Lynch *et al.*, 1992), consistent with its synthesis being independent of malonyl-CoA. As adipose tissue mobilization declines, due to increasingly positive energy balance and depletion of stored tissue, the proportions of short-chain and medium-chain fatty acids in milk fat increase. The time required for



**Figure 2.6.** Proportions of individual fatty acids in milk fat at 1, 4, 8 and 12 weeks of lactation relative to their proportions at 16 weeks (from Palmquist *et al.*, 1993, *J. Dairy Sci.* **76**, 1753–1771).

stabilization of milk fatty acid composition depends on the amount of stored fat, milk fat yield, energy balance and the quantity of fat in the diet. The effect on the proportion of  $C_{14:0}$  in milk fat of supplementing dietary fat to lactating cows at parturition or delayed to the sixth week of lactation is shown in Figure 2.7. Without fat supplementation, the proportion of  $C_{14:0}$



**Figure 2.7.** Changes in the proportion of  $C_{14:0}$  in milk fat of cows fed a control diet (—+—) or added fat beginning at parturition (—△—) or 6th week of lactation (—○—). (From Palmquist *et al.*, 1993, *J. Dairy Sci.* **76**, 1753–1771).

increased until 8 weeks of lactation; with fat supplementation, the maximum proportion of C<sub>14:0</sub> was lower.

## 2.9. Effects of Dietary Fat on the Composition of Milk Fat

### 2.9.1. Effects of Low-fat Diets

Very low-fat diets reduce milk and fat yields (Maynard and McCay, 1929; Banks *et al.*, 1976) and greatly reduce the proportions and yields of the C<sub>18</sub> fatty acids, with the proportions of C<sub>16:0</sub> approaching 50% of the total fat yield (Virtanen, 1966; Banks *et al.*, 1976).

Increasing the C<sub>18</sub> content of low-fat diets resulted in a linear increase of C<sub>18</sub> fatty acids in the milk fat:

$$y = 75 + 0.54x$$

where  $y$  = total C<sub>18</sub> fatty acids in milk fat (g/d) and  $x$  = total C<sub>18</sub> fatty acid intake (g/d). Thus, dietary C<sub>18</sub> fatty acids were transferred to milk fat with 54% efficiency (Banks *et al.*, 1976). This is consistent with other estimates of a maximum transfer of 60%, assuming 80% digestibility of dietary fat (Palmquist, 1991) and 75% uptake of absorbed lipoprotein TAGs by the mammary glands (Palmquist and Mattos, 1978). Banks *et al.* (1976) also estimated the transfer of C<sub>16</sub> from diet to milk fat to be 93%; this exceptionally high value could be attributed only to nearly complete uptake of absorbed TAGs by the mammary tissue; however, estimates of C<sub>16</sub> transfer are heavily confounded by effects of changing dietary fat intake on *de novo* synthesis of C<sub>16</sub>.

### 2.9.2. Effects of Specific Fatty Acids

Incorporation of dietary unsaturated fat into milk fat by ruminants is low because of the efficient ruminal biohydrogenation process (Jenkins, 1993). Nevertheless, dietary fatty acids have profound effects on milk fat composition that have led to a prodigious amount of literature in the past 20 years (for reviews see Sutton, 1989; Grummer, 1991; Palmquist *et al.*, 1993; Kennelly, 1996; Mansbridge and Blake, 1997; Chilliard *et al.*, 2000, 2001).

Acyl chain length (C<sub>16</sub> vs C<sub>18</sub>) influences the proportions of these in milk fat; the effects of C<sub>16</sub> are more subtle because of compensation by reduced *de novo* synthesis of C<sub>16:0</sub> when long-chain fatty acids are supplemented in the diet. Palmitic acid was increased from 45% of milk fatty acids to 53% when a high (68%) C<sub>16:0</sub> supplement was added to a low-fat diet.

Similarly, supplementing soy oil (90% C<sub>18</sub>) increased the total C<sub>18</sub> of milk fat from 25% to 60% of milk fatty acids. Yields of C<sub>6</sub> to C<sub>14</sub> were reduced by both supplements, whereas the yield of C<sub>16:0</sub> was increased by palm oil and reduced by soya oil (Banks *et al.*, 1976); these effects are typical (Noble *et al.*, 1969). Similarly, supplementing increasing amounts of coconut oil (high in C<sub>12:0</sub> and C<sub>14:0</sub>) increased the proportions of these in milk fat and reduced the proportions and yields of short-chain fatty acids and C<sub>16:0</sub> (Storry *et al.*, 1971).

Oleic acid was increased to 48% of total milk fatty acids by feeding oleamide as a rumen-protected source of oleic acid (Jenkins, 1998). The response was nearly linear up to 5% of supplement in the diet dry matter. Proportions of all *de novo*-synthesized milk fatty acids, except butyric, were reduced (Jenkins, 1999). LaCount *et al.* (1994) abomasally infused fatty acids from canola or high oleic acid sunflower oil into lactating cows. The transfer of oleic acid to milk fat was linear (slope = 0.541; 0–350 g infused/day); the proportion of oleic acid in milk fat increased and proportions of all *de novo*-synthesized fatty acids, except C<sub>4</sub> and C<sub>6</sub> decreased. The proportion of C<sub>18:0</sub> also was unchanged. Linoleic acid from canola also was transferred linearly (slope = 0.527; 0–90 g infused/day). These transfers from the intestine are nearly identical to that reported by Banks *et al.* (1976). Hagemester *et al.* (1991) reported 42 to 57% transfer of abomasally-infused linolenic acid to milk fat.

Mammary uptake of individual fatty acids from plasma was explored by Enjalbert *et al.* (1998). Uptakes of palmitic, stearic and oleic acids were similar and nearly linear in the range of 400 to 750  $\mu$ molar in the plasma. As uptake increased, mammary balance (milk content minus uptake) of butyric acid increased linearly and the mean fatty acid chain length of synthesized fatty acids decreased linearly. Desaturation of stearic acid increased linearly ( $y = 0.52x$ ,  $r^2 = 0.75$ ,  $P < 0.001$ ) as stearic acid uptake increased, and decreased linearly as mammary uptake of *trans* C<sub>18:1</sub> increased ( $y = 61.2 - 4.03x$ ,  $r^2 = 0.31$ ,  $P < 0.02$ ). Decreasing mean chain length of synthesized fatty acids and increasing desaturation with increasing long-chain fatty acid uptake were interpreted as compensating responses to maintain the fluidity of milk fat at body temperature. Maintenance of milk fat fluidity has been suggested as a basic physiological requirement in the regulation of milk fat synthesis (Timmen and Patton, 1988). An interesting exception to this is the report of Emanuelson *et al.* (1991) who fed heat-treated rapeseed to cows in late lactation. The stearic acid content of the milk fat was 28.8% of the total weight of fatty acids and the milk fat coalesced into floating butter globules immediately upon milking. The levels of palmitic (21.6%) and oleic (28.8%) acids were within normal ranges, but the proportions of short-chain and medium-chain fatty acids were rather low. The authors were unable to repeat the phenomenon.

### 2.9.3. Feeding for Specific Milk Fatty Acid Profiles

A very large body of literature focused on feeding effects on the composition of milk fat has been published in recent years, driven by the increasing public interest in, and concern for, the role of fat in the human diet. Most of the feeding studies have involved manipulation of the type and amount of fat in the ration of lactating cows as the experimental approach. In many studies, the role of ruminal biohydrogenation or its manipulation has been a major focus. Therefore, the effects, but not the regulation, of ruminal biohydrogenation will be addressed. Issues addressed in these studies have included: (1) increased unsaturation/polyunsaturation; (2) reduced saturation; (3) increased n-3/fish oil fatty acids; and (4) increased CLA content of milk fat.

The earliest efforts to modify the composition of milk fat used an insoluble formaldehyde-crosslinked protein to encapsulate unsaturated vegetable oils. In numerous studies using this approach, linoleic acid was increased to as high as 35%, w/w, of the total milk fatty acids (reviewed by McDonald and Scott, 1977). Bitman *et al.* (1973) fed increasing amounts of safflower oil encapsulated in formaldehyde-treated casein. The content of milk fat increased linearly from 3.5 to 4.6% as supplemental protected oil was increased from 0 to 1320 g/day per cow. The concentration of linoleic acid increased to 33% of total milk fatty acids, with a compensating decrease in C<sub>16:0</sub> and a smaller decrease in C<sub>14:0</sub>. The concentration of milk fat decreased to lower than pretreatment levels when the supplement was removed, a common observation (Pan *et al.*, 1972). A typical milk fatty acid profile from cows fed a protected sunflower/soybean (70/30) supplement is shown in Table 2.1.

Though feeding protected polyunsaturated fats has been instrumental in developing an understanding of the regulation of milk fat synthesis, it has not found practical application. In addition to increasing the cost of feeding, it has been difficult to assure product quality (consistency of protection); government and the public have been reluctant to approve formaldehyde as a component of feed ingredients because some amino acids may be transformed to potential carcinogens; and, importantly, highly polyunsaturated milk fat has very poor oxidative stability and its physical properties are not well suited for processed products (McDonald and Scott, 1977). Treatment of feedstuffs with formaldehyde is now more widely accepted, and attention has turned to using this method to protect high oleate oilseeds. Ashes *et al.* (1992) fed 0.52 kg/d of protected canola seeds to lactating cows. Milk fat percentage and yield were increased without changing milk yield. The proportions of C<sub>14:0</sub> and C<sub>16:0</sub> were reduced by 20 and 25%, respectively, whereas proportions of C<sub>18:0</sub>, C<sub>18:1</sub>, C<sub>18:2</sub> and C<sub>18:3</sub> were increased by 30,

**Table 2.1.** Fatty acid composition (weight % of total fatty acids) of milk fat from cows fed a standard diet or supplemented with protected oilseeds

Fatty acid	Control <sup>a</sup>	Sunflower/soybean <sup>a</sup>	Canola <sup>b</sup>
C <sub>4</sub>	3.2	2.8	3.2
C <sub>6</sub>	2.2	1.4	2.4
C <sub>8</sub>	1.1	0.8	1.9
C <sub>10:0</sub>	3.6	1.7	3.2
C <sub>12:0</sub>	3.9	1.7	3.6
C <sub>14:0</sub>	11.4	5.9	9.5
C <sub>15:0</sub>	2.1	0.9	—
C <sub>16:0</sub>	25.9	15.2	19.9
C <sub>16:1</sub>	2.7	0.5	3.3
C <sub>18:0</sub>	10.9	14.0	9.2
C <sub>18:1</sub>	28.6	37.6	29.2
C <sub>18:2</sub>	3.0	16.6	4.9
C <sub>18:3</sub>	1.0	0.9	2.6

<sup>a</sup> Calculated from Barbano and Sherbon (1980). Formaldehyde-protected sunflower/soybean (70/30); 1250 g oil/day.

<sup>b</sup> Ashes *et al.* (1992). Formaldehyde-protected canola; 520 g oil/day.

22, 122 and 62%, respectively. Relatively small changes were observed for short-chain and medium-chain fatty acids.

Several other procedures have been developed to protect unsaturated fatty acids from ruminal biohydrogenation. Of these, only the amide derivative has extensive research documentation (Jenkins, 1998, 1999), but has not been applied commercially. Often, calcium soaps of palm oil or canola fatty acids are referred to as “protected.” These are not protected from ruminal biohydrogenation (Table 2.2), but rather are ruminally inert with regard to their effects on the rumen microbial population.

#### 2.9.4. Supplementation with Oilseeds and Commercial Fats

Numerous types of fat are available commercially as supplemental energy sources. Many are products of the rendering industry and include tallow, lard (pork) and poultry fats. Recycled cooking oils from the restaurant industry are used also, usually as a blend with animal fats or oils from the food oil refining industry. Generally, unsaturated oils are undesirable as energy supplements for lactating cows (Palmquist and Jenkins, 1980; Jenkins, 1993). Tallow has long been a staple energy source for dairy diets; however, recent research (Onetti *et al.*, 2002) suggests that supplemental tallow may reduce milk fat percent modestly, which was attributed to increased concentrations of *trans*-10 C<sub>18:1</sub> in the milk fat (see Section 2.9.5

**Table 2.2.** Fatty acid composition of milk fat from cows fed various fat supplements

	Calcium salts						Whole roasted soybeans	Tallow	Fish oil
	None	Palm oil	Palm oil	Canola	Soy	Linseed			
% of feed DM	–	3.0	1.0	5.0	4.9	4.3	4.6	5.4	2.0
Reference	a	a	b	c	c	c	d	d	e
Fatty acid	weight % of reported fatty acids								
C <sub>4:0</sub>	3.25	3.15	3.43	4.52	4.65	4.92	3.65	2.65	3.88
C <sub>6:0</sub>	2.28	1.92	2.23	2.33	2.39	2.89	2.34	1.59	2.66
C <sub>8:0</sub>	1.57	1.24	1.27	1.16	1.14	1.52	1.31	0.83	1.30
C <sub>10:0</sub>	3.36	2.31	2.88	2.59	2.34	3.09	2.77	1.75	2.83
C <sub>12:0</sub>	4.11	2.92	3.19	2.53	2.21	2.71	3.03	2.23	3.16
C <sub>14:0</sub>	13.13	10.35	10.09	9.39	8.10	9.20	9.75	10.70	11.40
C <sub>14:1c-9</sub>	0.95	0.74	–	1.10	0.74	0.71	1.05	1.73	0.77
C <sub>15:0</sub>	1.10	0.82	–	1.06	0.84	0.96	–	–	0.98
C <sub>16:0</sub>	32.58	34.75	30.25	19.26	19.25	19.09	25.44	31.68	27.56
C <sub>16:1c-9</sub>	1.83	2.18	1.59	1.20	0.90	0.84	1.33	3.08	1.40
C <sub>18:0</sub>	10.74	10.50	10.74	15.04	14.76	14.99	12.79	9.40	8.11
C <sub>18:1c-9</sub>	20.23	24.60	23.80	26.95	25.99	22.92	21.88	23.73	15.08
C <sub>18:1r-11</sub>	–	–	–	8.42	12.59	10.18	3.95	4.96	2.34
C <sub>18:2</sub>	2.70	2.76	4.09	2.32	2.45	3.30	5.27	2.36	2.20
C <sub>18:3</sub>	1.28	1.13	0.47	0.20	0.19	0.31	1.00	0.43	0.85
C <sub>18:2c-9, t-11</sub>	–	–	0.49	–	–	–	1.03	0.81	0.88
C <sub>18:2r-10, c-12</sub>	–	–	0.03	–	–	–	–	–	0.04
C <sub>20:5 n-3</sub>	–	–	–	–	–	–	–	–	0.24
C <sub>22:5 n-3</sub>	–	–	–	–	–	–	–	–	0.28
C <sub>22:6 n-3</sub>	–	–	–	–	–	–	–	–	0.26

<sup>a</sup> Schauff and Clark (1992)  
<sup>b</sup> Giesy *et al.* (2002)  
<sup>c</sup> Chouinard *et al.* (1998)  
<sup>d</sup> Morales *et al.* (2000)  
<sup>e</sup> AbuGhazaleh *et al.* (2002)

milk fat depression). Increasing the proportion of corn silage in dietary forage (reducing alfalfa silage) also increased the content of *trans*-10 C<sub>18:1</sub> and lowered milk fat percent (Onetti *et al.*, 2002) (Table 2.3).

Calcium soaps of palm oil fatty acids are used widely as an energy supplement. With a content of 45 to 50% C<sub>16:0</sub>, these calcium soaps increase palmitic acid in milk fat compared with oilseeds (Table 2.2). Whole oilseeds (cottonseed, canola, soybeans) also are used widely as energy supplements in dairy diets. All except cottonseed oil (25% C<sub>16:0</sub>) contain predominantly C<sub>18</sub> fatty acids. When fed whole or crushed, the oil tends to be released slowly,

**Table 2.3.** Least square means for fatty acid composition of milk fat when decreasing proportions of corn silage were fed without (0%) or with (2%) tallow<sup>a</sup>

Corn silage <sup>c</sup>	0% Tallow			2% Tallow			Significance (P) <sup>b</sup>	
	50%	37.5%	25%	50%	37.5%	25%	F	L
Fatty Acid	(g/100 g of fatty acids)							
C <sub>4:0</sub> to C <sub>14:0</sub>	25.3	24.6	24.7	21.4	21.1	21.4	0.001	NS
C <sub>16:0</sub>	28.9	29.1	29.1	28.1	27.9	27.8	0.01	NS
C <sub>18:0</sub>	8.1	8.7	8.7	8.2	9.1	9.2	NS	0.01
C <sub>18:1</sub>	23.2	23.3	23.0	26.3	26.1	25.5	0.001	NS
C <sub>18:1</sub> isomers								
<i>trans</i> -6/8	0.37	0.39	0.37	0.56	0.54	0.50	0.001	NS
<i>trans</i> -9	0.51	0.56	0.49	0.72	0.65	0.71	0.001	NS
<i>trans</i> -10	1.3	1.0	0.9	2.2	1.8	1.4	0.001	0.001
<i>trans</i> -11	1.1	1.1	1.0	0.94	1.1	1.0	NS	NS
<i>trans</i> -12	0.41	0.41	0.42	0.42	0.50	0.52	0.01	0.04
<i>trans</i> -16	0.06	0.05	0.05	0.03	0.04	0.05	NS	NS
<i>cis</i> -9	17.8	18.2	17.8	20.0	19.8	19.4	0.001	NS
<i>cis</i> -11	0.87	0.92	0.93	0.94	0.94	0.95	0.04	NS
<i>cis</i> -12	0.41	0.44	0.52	0.22	0.33	0.44	0.001	0.001
C <sub>18:2e9t11</sub>	0.60	0.57	0.58	0.61	0.63	0.62	0.06	NS
C <sub>18:2t10c12</sub>	0.01	0.01	0.02	0.01	0.02	0.02	NS	NS
C <sub>18:2</sub>	4.5	4.5	4.5	4.2	4.2	4.5	NS	NS
C <sub>18:3</sub>	0.33	0.40	0.50	0.28	0.39	0.45	0.01	0.001
Other	8.8	8.2	8.4	10.3	10.1	9.5	0.01	NS

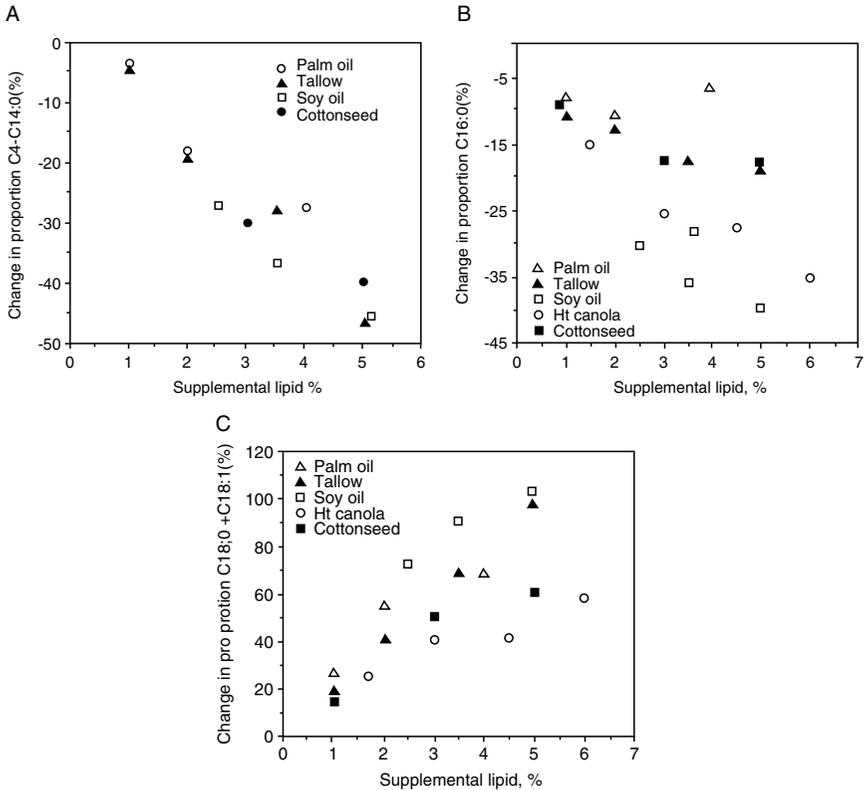
<sup>a</sup> Onetti *et al.* (2002).<sup>b</sup> F = Main effect of fat; L = linear effect of forage.<sup>c</sup> Diets: 50% forage and 50% concentrate (DM). Forages were: (1) 50% of diet DM as corn silage, (2) 37.5% corn silage and 12.5% alfalfa silage, and (3) 25% corn silage and 25% alfalfa silage.

with nearly complete biohydrogenation of the unsaturated fatty acids. All increase the proportion of C<sub>18:1</sub> in milk fat and reduce the proportions of C<sub>6</sub> to C<sub>16</sub> fatty acids, but especially C<sub>14:0</sub> and C<sub>16:0</sub>. C<sub>18:0</sub> in milk fat is often quite high when canola (Chouinard *et al.*, 1997; Ward *et al.*, 2002) or whole roasted soybeans (Morales *et al.*, 2000; Timmons *et al.*, 2001) are fed, apparently due to nearly complete ruminal biohydrogenation. Uniquely, feeding whole soybeans also increases the proportions of C<sub>18:2</sub> and C<sub>18:3</sub> in milk fat; apparently, some portion of the soybeans pass from the rumen undegraded, causing significant increases in the levels of those fatty acids in milk fat (Morales *et al.*, 2000), and thereby increasing the susceptibility of the milk fat to oxidative rancidity (Timmons *et al.*, 2001).

Increasing public concern related to the composition of dietary fats has increased interest in the n-3 fatty acid content of milk fat. The content of linolenic acid in milk fat from cows grazing pasture may be more than

double that from cows fed in stalls or feedlots (Dhiman *et al.*, 1999; Chilliard *et al.*, 2001) owing to the high content and rumen escape of  $C_{18:3}$  n-3 from forages. Others have investigated the transfer of the long-chain ( $C_{20}$  and  $C_{22}$ ) n-3 fatty acids of fish oil to milk fat. Adding 2% menhaden fish oil to the diet of lactating cows increased the content of  $C_{20:5}$  n-3 and  $C_{22:6}$  n-3 (EPA and DHA, respectively) from 0.05 and 0.04% in the control to 0.24 and 0.26% in milk fat of supplemented cows (AbuGhazaleh *et al.*, 2002). Feeding fish oil fatty acids in combination with vegetable oils synergistically increases the CLA content of milk fat (see Chapter 3).

Changes in the proportions of fatty acids in milk fat by supplementation of various oils and oilseeds are summarized in Figure 2.8 (Grummer, 1991). Hermansen (1995) developed a set of regression equations to predict the composition of milk fat based on the proportions of lauric, myristic and



**Figure 2.8.** Changes in proportions of milk fatty acids relative to control treatments with increasing supplementation of fats or oilseeds A.  $C_4$  to  $C_{14:0}$ ; B.  $C_{16:0}$ ; C.  $C_{18:0} + C_{18:1}$ . (From Grummer, 1991, *J. Dairy Sci.* **74**, 3244–3257).

palmitic acids in the dietary fat and on total dietary fat intake. The model effectively predicted milk fatty acid profile with respect to lauric, myristic, palmitic and oleic acids and total C<sub>18</sub> fatty acids across experiments. Predictions were less precise for short-chain acids, stearic acid and polyunsaturated fatty acids.

### 2.9.5. Low Milk Fat Syndrome

Low milk fat syndrome (milk fat depression) has been investigated intensively for more than 40 years. Early investigators pursued the link between changes in the ruminal acetate:propionate ratio and the % fat in milk (van Soest, 1963). A glucogenic response, whereby increased production of ruminal propionate would increase blood glucose and insulin concentrations, with decreased fatty acid release from adipose tissue, was proposed by McClymont and Vallance (1962), and developed further by van Soest (1963). Whereas intravenous infusion of glucose or glycerol (Vallance and McClymont, 1959), or duodenal infusion of glucose (Hurtaud *et al.*, 2000) have been shown to reduce milk fat percentage; these increase the relative proportion of *de novo*-synthesised fatty acids in milk fat (Hurtaud *et al.*, 2000), contrary to the consistent decrease in these in classical low milk fat syndrome (Bauman and Griinari, 2001). Thus, it became apparent that other aspects of lipid metabolism were involved (Davis and Brown, 1970). Recent research has suggested that the low milk fat syndrome is mediated by *trans*-10 C<sub>18:1</sub> (Griinari *et al.*, 1998) or by *trans*-10, *cis*-12 C<sub>18:2</sub> (Baumgard *et al.*, 2000), or both (Bauman and Griinari, 2001, 2003), that are products of changes in ruminal biohydrogenation in the presence of unsaturated fatty acids and a low ruminal pH (Griinari *et al.*, 1998). This research has not excluded the possibility that the actual effector(s) may be as yet unidentified fatty acid isomer(s) that are highly correlated with *trans*-10 C<sub>18:1</sub> or *trans*-10, *cis*-12 C<sub>18:2</sub>, or both (Bauman and Griinari, 2003). Depressed milk fat synthesis is associated with reduced enzyme activity or mRNA abundance, or both, for acetyl CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase, lipoprotein lipase and glycerol phosphate acyl transferase (Piperova *et al.*, 2000; Ahnadi *et al.*, 2002; Peterson *et al.*, 2003). Recent advances in regulation of the low milk fat syndrome have been reviewed (Bauman and Griinari, 2001, 2003).

### 2.10. Milk Fat Composition and Quality

The uniqueness of milk fat is not limited to its fatty acid profile. If the 400 fatty acids of milk fat were distributed randomly in the milk fat TAGs, the total theoretical number of glycerides would be  $64 \times 10^6$  (Jensen, 2002);

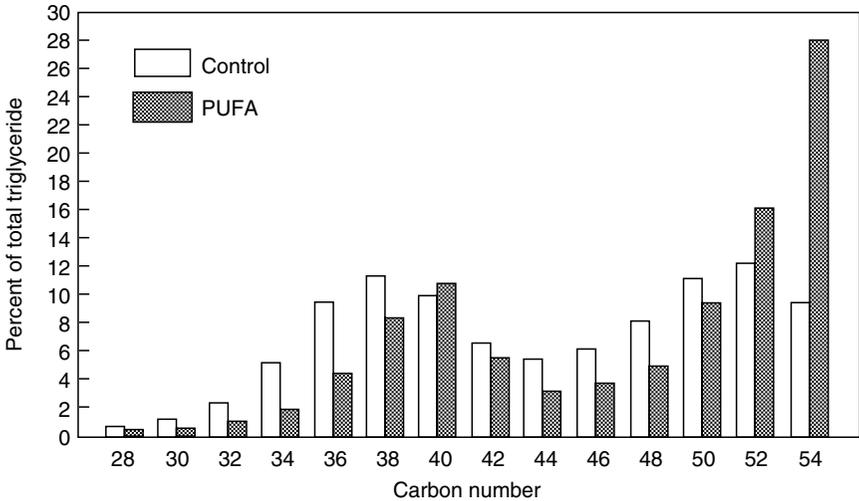
however, distribution is not random, as noted above (see *Triacylglycerol Synthesis*). The predominant locations of fatty acids in TAGs are shown in Table 2.4; however, distributions in high and low molecular weight TAGs can differ widely from the mean (Morrison and Hawke, 1977b). Also, 36% of TAGs were found to contain C<sub>4:0</sub> or C<sub>6:0</sub> and two long-chain fatty acids (Jensen, 2002). Jensen (2002) listed 22 TAG structures that were found at > 1 mol % in the milk fat; these totaled 42.7 mol %.

The acyl carbon number (CN; total carbon in the acyl chains) of milk fat TAGs typically ranges from 26 to 54. This distribution contributes significantly to the physical characteristics of plasticity and spreadability (functionality or rheological properties) of milk fat. This property is caused by a large proportion of the fat occurring in the molten state at room temperature, supported in a matrix of solid fat that makes up only a small percentage of the total fat (German *et al.*, 1997). Changing the fatty acid chain length in milk fat will change the acyl chain number; an extreme example is shown in Figure 2.9. In this case, most of the CN54 acyl chains were linoleic acid from feeding a protected lipid supplement (Morrison and Hawke, 1977a). Butter made from high-linoleic (>20%) milk fats is slower to churn, more susceptible to oxidation on storage, and breaks down with oiling-off at a temperature above 10°C. Cheeses made from milk with 10–12% fatty acids as C<sub>18:2</sub> were acceptable, whereas cheeses with a higher linoleic acid content had off-flavour, a soft body and a mealy texture (McDonald and Scott, 1977). More recent applications using milled rapeseed or calcium salts of oilseed fatty acids have led to the successful development of modified butter (Chouinard *et al.*, 1998; Fearon, 2001; Fearon *et al.*, 2004) and cheese (Jaros *et al.*, 2001).

**Table 2.4.** Predominant distribution (mole percent) of fatty acids in milk fat triacylglycerols<sup>a</sup>

Fatty acid	TAG position		
	sn-1	sn-2	sn-3
C <sub>4:0</sub>	1.6	0.3	98.1
C <sub>6:0</sub>	3.1	3.9	93.0
C <sub>8:0</sub>	10.3	55.2	34.5
C <sub>10:0</sub>	15.2	56.6	28.2
C <sub>12:0</sub>	23.7	62.9	13.4
C <sub>14:0</sub>	27.3	65.6	7.1
C <sub>16:0</sub>	44.1	45.4	10.5
C <sub>18:0</sub>	54.0	16.2	29.8
C <sub>18:1</sub>	37.3	21.2	41.5

<sup>a</sup> Adapted from Jensen (2002).



**Figure 2.9.** Distribution of triacylglycerols by acyl carbon number in milk fat from cows fed diets with no added fat (control) or high in polyunsaturated fats (PUFA). See Table 2.1 for fatty acid profile (from Palmquist *et al.*, 1993. *J. Dairy Sci.* **76**, 1753–1771).

## Acknowledgement

I acknowledge, with much appreciation, the careful and critical review of this manuscript by my former mentor, Dr. R.L. “Lee” Baldwin. His comments and additions, especially for the flow of carbon in intermediary metabolism, the generation of reducing equivalents and the regulation of acetyl-CoA carboxylase, are invaluable contributions to the authority and completeness of this chapter.

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**ABBREVIATIONS**

ACBP	Acyl CoA Binding Protein
ACC	Acetyl CoA Carboxylase
ACP	Acyl Carrier Protein
Akt	Serine/threonine protein kinase
AMP	Adenosyl Monophosphate
ATP	Adenosyl Triphosphate
cAMP	Cyclic AMP
CLA	Conjugated Linoleic Acid
CoASH	Coenzyme A
CMP	Cytidyl Monophosphate
CDP	Cytidyl Diphosphate
CTP	Cytidyl Triphosphate
DGAT	Diacyl Glycerol Acyl Transferase
DHA	Docosahexaenoic acid
DM	Dry Matter
EPA	Eicosapentaenoic acid
FABP	Fatty Acid Binding Protein
FAT	Fatty Acid Translocator
GLUT	Glucose Transporter
GPAT	Glycerol Phosphate Acyl Transferase
IGF	Insulin-like Growth Factor
LPL	Lipoprotein Lipase
NADH + H <sup>+</sup>	Reduced Nicotine Adenine Dinucleotide
NADPH + H <sup>+</sup>	Reduced Nicotine Adenine Dinucleotide Phosphate
NEFA	Non-esterified Fatty Acids (also called “free” fatty acids, FFA)
OAA	Oxaloacetate
PFK	Phosphofructokinase
PPi	Inorganic Pyrophosphate
PPP	Pentose phosphate pathway
SCD	Stearoyl-CoA Desaturase
sn	Sterosppecific numbering (of positions on asymmetric glycerol)
SREBPs	Sterol Receptor Element-Binding Proteins
TAG	Triacylglycerol
VLDL	Very Low Density Lipoproteins

# Conjugated Linoleic Acid: Biosynthesis and Nutritional Significance

D.E. Bauman and A.L. Lock

## Abstract

The term conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid with a conjugated double bond system; milk fat can contain over 20 different isomers of CLA. CLA isomers are produced as transient intermediates in the rumen biohydrogenation of unsaturated fatty acids consumed in the diet. However, *cis*-9, *trans*-11 CLA, known as rumenic acid (RA), is the predominant isomer (up to 90% of total) because it is produced mainly by endogenous synthesis from vaccenic acid (VA). VA is typically the major biohydrogenation intermediate produced in the rumen and it is converted to RA by  $\Delta^9$ -desaturase in the mammary gland and other tissues.

Biomedical studies with animal models have shown that RA as well as VA have anticarcinogenic and antiatherogenic properties, with the effects of VA being related to its conversion to RA. The anticarcinogenic effects have been observed for a wide range of cancer types, but the most impressive results have been reported in relation to mammary cancer. Of special importance, RA and VA are potent anticarcinogens when supplied as natural food components in the form of VA/RA-enriched butter. The functional food considerations of CLA isomers in dairy products realistically relate only to RA as the major isomer, although this should include VA because in humans it serves for the endogenous synthesis of RA. The RA and VA content in milk fat are directly related and they can be markedly enhanced through the use of diet formulation and nutritional management of dairy cows.

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

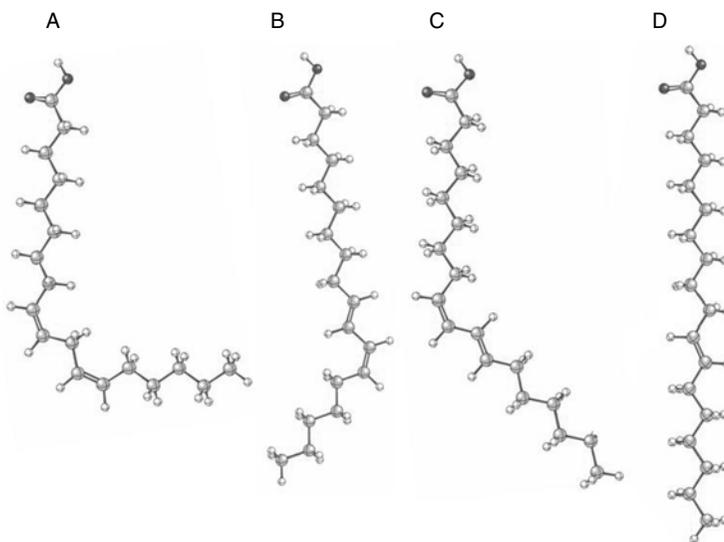
*Trans-10, cis-12* CLA is another CLA isomer in milk fat which can affect lipid metabolism. It is generally present at low concentrations in milk fat (typically <0.2% of CLA); under some dietary conditions, a portion of the rumen biohydrogenation shifts to produce more of this isomer, although it is still only a minor portion of total CLA. These dietary conditions are associated with milk fat depression and as little as 2 g/d of *trans-10, cis-12* leaving the rumen will reduce milk fat synthesis by 20%. Because of the potency and specificity of this CLA isomer, it is being developed as a dairy management tool to allow for a controlled reduction in milk fat output.

CLA isomers in milk fat and how they relate to both animal agriculture and human health are rapidly expanding fields. Milk and dairy products offer exciting opportunities in the area of functional foods, and the functional properties of VA and RA in milk further serve to illustrate the value of dairy products in the human diet.

### 3.1. Introduction

An adequate supply of good-quality food is essential for human health and well-being. Milk and meat products derived from ruminants represent important sources of nutrients in human diets, providing energy, high quality protein, and essential minerals and vitamins (National Research Council, 1988; Demment and Allen, 2003). Nutritional quality is increasingly an important consideration in food choices because of the growing consumer awareness of the link between diet and health. Many foods contain micro-components that have beneficial effects beyond those associated with their traditional nutrient content, and these are often referred to as “functional food” components. One such component in foods derived from ruminants is conjugated linoleic acid (CLA).

CLA refers to a mixture of positional and geometric isomers of linoleic acid (*cis-9, cis-12* octadecadienoic acid) with a conjugated double bond system. The structure of two CLA isomers is contrasted with linoleic and vaccenic acids in Figure 3.1. The presence of CLA isomers in ruminant fat is related to the biohydrogenation of polyunsaturated fatty acids (PUFAs) in the rumen. Ruminant fats are relatively more saturated than most plant oils and this is also a consequence of biohydrogenation of dietary PUFAs by rumen bacteria. Increases in saturated fatty acids are considered undesirable, but consumption of CLA has been shown to be associated with many health benefits, and food products derived from ruminants are the major dietary source of CLA for humans. The interest in health benefits of CLA has its genesis in the research by Pariza and associates who first demonstrated that



**Figure 3.1.** Chemical structures of linoleic acid (*cis*-9, *cis*-12 18:2; A), *trans*-10, *cis*-12 conjugated linoleic acid (B), ruminic acid (*cis*-9, *trans*-11 conjugated linoleic acid; C) and vaccenic acid (*trans*-11 18:1; D).

CLA isomers are functional food components when their search for mutagens in cooked meat instead identified CLA as an antimutagen (see Pariza, 1999). As a result of this discovery, research on CLA has increased exponentially over the last decade and a number of potential health benefits of CLA have been reported. The anticarcinogenic activity of CLA has been established clearly, but biomedical studies with animal models have identified an impressive range of additional positive health effects for CLA as summarized in Chapter 17. Particularly noteworthy is the fact that CLA is a potent anticarcinogen when supplied as a natural food component in the form of CLA-enriched butter as discussed later in this review.

The presence of CLA in ruminant milk has been known for more than 70 years and in this chapter we will first review the dietary sources of CLA and provide an overview of the analytical challenges associated with quantifying CLA in foods and biological samples. Secondly, we will review the origin of the different CLA isomers present in milk fat, developing the interrelationships between biohydrogenation intermediates produced in the rumen, synthesis of CLA in the tissues and the presence of these isomers in milk fat. Thirdly, we will highlight the nutritional and physiological factors

that effect the level of CLA in milk fat and discuss milk quality considerations for dairy products that have a naturally enhanced content of CLA. Finally, we will review the biological effects of CLA related to the dairy cow and dairy products. This will include its effects on milk fat synthesis in dairy cows, an area that has progressed rapidly and promises to contribute to our general understanding of the regulation of lipid metabolism. Our review of the biological effects will also include the significance of CLA in dairy products and its potential as a functional food component that benefits human health. Regular updates and references on the biology of CLA in dairy chemistry can be found at [www.wisc.edu/fri/clarefs.htm](http://www.wisc.edu/fri/clarefs.htm) and [www.ansci.cornell.edu/bauman](http://www.ansci.cornell.edu/bauman).

### 3.2. Dietary Sources

The predominant source of CLA in human diets is ruminant-derived food products. CLA is a fatty acid so it is present in milk fat and muscle fat. In the U.S., dairy products provide about 70% of the intake of CLA and beef products account for another 25% (Ritzenthaler *et al.*, 2001). Similar values for the contribution of different food classes have been reported for other countries (see Parodi, 2003).

Scientists at the University of Reading, UK, first demonstrated that fatty acids obtained from summer butter differed from those obtained from winter butter by exhibiting a much stronger spectrophotometric absorption at 230  $\mu\text{m}$  (Booth *et al.*, 1933). It was subsequently concluded that the adsorption at this wavelength was due to a conjugated double bond pair (Moore, 1939). Parodi (1977) was the first to identify *cis*-9, *trans*-11 octadecadienoic acid as a fatty acid in milk fat that contained the conjugated double bond pair. As analytical techniques improved it was discovered that milk fat and body fat from ruminants contained many isomers of CLA that differ by position (e.g., 7–9, 8–10, 9–11, 10–12, 11–13) or geometric orientation (*cis-trans*, *trans-cis*, *cis-cis*, and *trans-trans*) of the double bond pair. The range of CLA isomers and their levels in milk and dairy products is summarized in Table 3.1. *Cis*-9, *trans*-11 is the major CLA isomer in ruminant fat, representing about 75 to 90% of the total CLA, and the common name of “ruminic acid” (RA) has been proposed for this isomer because of its unique relationship to ruminants (Kramer *et al.*, 1998). The second most common isomer is *trans*-7, *cis*-9 CLA, representing about 10% of total CLA. Each of the other CLA isomers is at a low concentration when present, generally representing less than 0.5% of the total CLA in ruminant fat.

**Table 3.1.** Range of positional and geometric isomers of conjugated C<sub>18:2</sub> fatty acids in milk and dairy products. Adapted from Lock and Bauman (2004)\*

Isomer	% of total CLA isomers
<i>trans</i> -7, <i>cis</i> -9	1.2–8.9
<i>trans</i> -7, <i>trans</i> -9	<0.1–2.4
<i>trans</i> -8, <i>cis</i> -10	<0.1–1.5
<i>trans</i> -8, <i>trans</i> -10	0.2–0.4
<i>cis</i> -9, <i>trans</i> -11	72.6–91.2
<i>trans</i> -9, <i>trans</i> -11	0.8–2.9
<i>trans</i> -10, <i>cis</i> -12	<0.1–1.5
<i>trans</i> -10, <i>trans</i> -12	0.3–1.3
<i>cis</i> -11, <i>trans</i> -13	0.2–4.7
<i>trans</i> -11, <i>cis</i> -13	0.1–8.0
<i>trans</i> -11, <i>trans</i> -13	0.3–4.2
<i>cis</i> -12, <i>trans</i> -14	<0.01–0.8
<i>trans</i> -12, <i>trans</i> -14	0.3–2.8
<i>cis-cis</i> isomers	0.1–4.8

\* Data derived from seven studies where fatty acid analysis was carried out on milk samples (Precht and Molkenkin, 1997; Piperova *et al.*, 2002; Kraft *et al.*, 2003; Shingfield *et al.*, 2003; Kay *et al.*, 2004), butter (Bauman *et al.*, 2000) or cheese (Rickert *et al.*, 1999).

### 3.3. Analytical Challenges

Biological samples generally contain multiple isomers of CLA, many at very low concentrations, and each may differ in their biological effects. Thus, the ability to determine the concentration of specific isomers is becoming increasingly important, and frequently a combination of analytical methods is required to quantify fully CLA isomers and related fatty acids (Christie, 2003; Kramer *et al.*, 2004). The analysis of CLA typically requires their conversion to derivatives that can be separated from other fatty acids in the sample, usually by either gas chromatography (GC) or high-performance liquid chromatography (HPLC). Early investigations generally used high temperature, acid-catalyzed methylation to prepare fatty acid methyl esters, but subsequent work established that this procedure causes extensive isomerization, producing mainly *trans/trans* isomers. Therefore, data on the distribution of CLA isomers from early investigations are highly questionable (Yurawecz *et al.*, 1999).

GC provides the basis for most analytical approaches reported in the literature, and the use of alkali-catalysed methylation has proven to be the most accurate method for analysis of CLA (Yurawecz *et al.*, 1999). Sodium methoxide is the catalyst used most widely and has the advantage that it does not isomerize conjugated double bonds or form methoxy artifacts.

Kramer *et al.* (1997) demonstrated that this procedure completely methylated test samples containing mainly triglycerides, but it did not methylate free fatty acids. Since milk fat consists of ~98% triglycerides, the contribution made by other lipids can often be disregarded so that only a base-catalyzed methylation is necessary (Yurawecz *et al.*, 1999). The transmethylation procedure described by Christie (1982) with modifications by Chouinard *et al.* (1999a) to minimize the loss of highly-volatile short chain fatty acids, is used widely and recommended for the analysis of fatty acids in milk. In the GC analysis, the type of column is another important consideration. Long (100–120 m) highly polar columns are used typically and offer a reasonable degree of separation for CLA isomers, and especially *trans* 18:1 fatty acids (Christie, 2003). However, with typical GC procedures, *trans*-7, *cis*-9 CLA and RA co-elute, and other CLA isomers are often not separable, especially if there is a low concentration of one isomer relative to another.

Analysis of the CLA content and profile of animal tissues or biological fluids containing a mixture of lipid classes is more difficult. In order for all of the fatty acids to be methylated, a two-stage methylation procedure is recommended. Kramer *et al.* (1997) evaluated many different combinations of acid/base catalysts and concluded that the best compromise was the use of sodium methoxide followed by a mild acidic methylation, which resulted in the methylation of the majority of the fatty acids with minimal isomerization of the CLA isomers. However, mild boron trifluoride or 1% methanolic sulphuric acid with a minimal temperature and reaction time are often used with good success.

Additional analytical methods are appropriate when a more complete characterization of the CLA isomers in biological samples is required. Most often, a combination of GC and silver ion-HPLC is used and permits excellent separation and identification of positional and geometrical isomers of CLA (see Adlof, 2003, and Kramer *et al.*, 2004, for detailed reviews of this approach). In addition, the use of gas chromatography-mass spectrometry (GC-MS) has become increasingly popular and represents a very powerful technique for identification of the position of double bonds in fatty acids (see Dobson, 2003), and the orientation of those bonds in CLA isomers (Michaud *et al.*, 2003).

In summary, the analysis of CLA can be simple or extensive. The particular objectives and the anticipated use of the analytical data will determine the extent to which individual CLA isomers need to be separated, identified and quantified (Christie, 2003). Methodology for the analysis of CLA and related fatty acids continues to evolve and it is recommended that the reader consult recent reviews and publications in this area before undertaking such analysis for the first time. We recommend Christie (2003) and Kramer *et al.* (2004) as excellent practical guides on the analysis of CLA.

In addition, periodic updates on CLA analysis can be found at [www.lipidlibrary.co.uk](http://www.lipidlibrary.co.uk).

### 3.4. Origin of CLA in Milk Fat

#### 3.4.1. Lipid Metabolism in the Rumen

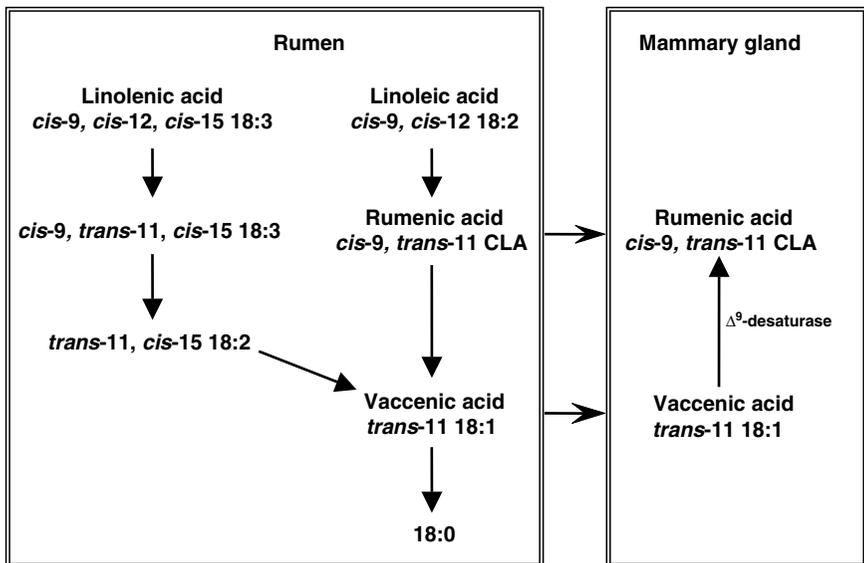
The diet of lactating dairy cows typically contains 4 to 5% fat, with the major PUFAs being linoleic and linolenic acids that are supplied mainly from dietary concentrates and forages, respectively. When dietary lipids enter the rumen, the initial step is hydrolysis of the ester linkages in the triglycerides, phospholipids, and glycolipids. Hydrolysis of dietary lipids in the rumen involves extracellular lipases that are produced by the rumen bacteria; there is little evidence to support significant roles for rumen protozoa and fungi, or salivary or plant lipases in rumen hydrolysis. The extent of hydrolysis of dietary lipids in the rumen is generally high (>85%), and a number of factors that affect the rate and extent of hydrolysis have been identified (see Harfoot, 1981; Doreau and Ferlay, 1994; Doreau *et al.*, 1997b; Harfoot and Hazlewood, 1997 for reviews).

Biohydrogenation of PUFAs is the second major transformation that dietary lipids undergo in the rumen and this process first requires that the fatty acid is free. As a consequence, rates are always less than those of hydrolysis and factors that affect hydrolysis also affect rates of biohydrogenation. In the 1960s and 1970s, an extensive series of *in vitro* and *in vivo* studies examined rumen biohydrogenation (see Dawson and Kemp, 1970; Keeney, 1970; Harfoot, 1981; Harfoot and Hazlewood, 1997). Most biohydrogenation (>80%) occurs in association with the small dense (fine) food particles and this has been attributed to extracellular enzymes of bacteria either associated with the feed or free in suspension. A few species of rumen bacteria capable of carrying out the biohydrogenation reactions have been identified and predominant pathways have been elucidated. Kemp and Lander (1984) classified rumen bacteria involved in biohydrogenation into two groups based on their metabolic pathways. Group A included bacteria that could hydrogenate 18 carbon PUFAs to *trans* 18:1 fatty acids, whereas only a few species, characterized as Group B, could hydrogenate *trans* 18:1 fatty acids to stearic acid (Harfoot and Hazlewood, 1997). Thus, complete biohydrogenation of unsaturated fatty acids generally requires bacteria from both groups. However, recent research has identified an occasional exception where a specific bacterial strain can carry out the complete biohydrogenation of PUFAs to C<sub>18:0</sub> (see Palmquist *et al.*, 2005).

The initial step in rumen biohydrogenation of linoleic and linolenic acids involves an isomerization of the *cis*-12 double bond to a *trans*-11

configuration, resulting in a conjugated dieonic or trienoic fatty acid (Figure 3.2). Next, is a reduction of the *cis*-9 double bond resulting in a *trans*-11 octadecenoic acid. Therefore, RA is an intermediate formed only during the biohydrogenation of linoleic acid. The conversion of RA to vaccenic acid (*trans*-11 18:1; VA) is catalyzed by a reductase. The structure of VA is given in Figure 3.1 and it is noteworthy that it is an intermediate in the biohydrogenation of both linoleic and linolenic acids (Figure 3.2). The final step is a further reduction of the *trans*-monoenes, producing stearic acid. Reduction of *trans*-octadecenoic fatty acids to stearic acid is generally the rate-limiting step and, as a consequence, there is often an accumulation of *trans* fatty acids in the rumen (Keeney, 1970).

As analytical techniques improved, we have gained an appreciation of the complexity of the biohydrogenation processes occurring in the rumen. In addition to the major pathways involving RA and VA as intermediates, there must be many additional pathways. A remarkable range of *trans* 18:1 and CLA isomers are produced during biohydrogenation and their outflow from the rumen based on limited data from growing cattle and lactating cows are shown in Table 3.2. This range of CLA and *trans*-18:1 isomers is



**Figure 3.2.** Pathways for ruminal and endogenous synthesis of rumenic acid (*cis*-9, *trans*-11 CLA) in the lactating dairy cow. Pathways for biohydrogenation of linoleic and linolenic acids yielding vaccenic acid (*trans*-11 18:1) are shown in the rumen box and endogenous synthesis by  $\Delta^9$ -desaturase is shown in the mammary gland box. Adapted from Bauman *et al.* (2003).

**Table 3.2.** Range of double bond positions in *trans* C<sub>18:1</sub> and conjugated C<sub>18:2</sub> fatty acids and their ruminal outflow in growing and lactating cattle\*

<i>Trans</i> 18:1		Conjugated 18:2	
Isomer	Ruminal outflow (g/day)	Isomer	Ruminal outflow (g/day)
<i>trans</i> -4	0.5–0.7	<i>trans</i> -7, <i>cis</i> -9	<0.01
<i>trans</i> -5	0.4–0.6	<i>trans</i> -7, <i>trans</i> -9	<0.01–0.05
<i>trans</i> -6–8	0.4–6.7	<i>trans</i> -8, <i>cis</i> -10	0.01–0.02
<i>trans</i> -9	0.8–6.2	<i>trans</i> -8, <i>trans</i> -10	<0.01–0.10
<i>trans</i> -10	1.7–29.1	<i>cis</i> -9, <i>cis</i> -11	<0.01–0.01
<i>trans</i> -11	5.0–121.0	<i>cis</i> -9, <i>trans</i> -11	0.19–2.86
<i>trans</i> -12	0.5–9.5	<i>trans</i> -9, <i>trans</i> -11	0.22–0.55
<i>trans</i> -13 + 14	6.5–22.9	<i>trans</i> -10, <i>cis</i> -12	0.02–0.32
<i>trans</i> -15	3.2–8.5	<i>trans</i> -10, <i>trans</i> -12	0.05–0.06
<i>trans</i> -16	3.1–8.0	<i>cis</i> -11, <i>trans</i> -13	0.01–0.10
		<i>trans</i> -11, <i>cis</i> -13	0.01–0.46
		<i>trans</i> -11, <i>trans</i> -13	0.09–0.40
		<i>cis</i> -12, <i>trans</i> -14	<0.01–0.05
		<i>trans</i> -12, <i>trans</i> -14	0.08–0.19

\* Data derived from three studies where samples were collected from the duodenum (Duckett *et al.*, 2002; Piperova *et al.*, 2002) or omasum (Shingfield *et al.*, 2003).

not accounted for by known pathways of rumen biohydrogenation. Isomerase is the enzyme that catalyses the key step that introduces the conjugated double bond system and, unfortunately, this enzyme has been studied in only a few species of rumen bacteria (Kepler and Tove, 1967; Kepler *et al.*, 1970; Yokoyama and Davis, 1971). The isomerase from *Butyrivibrio fibrisolvens* is a particulate enzyme bound to the bacterial cell membrane and it has an absolute substrate requirement for a *cis*-9, *cis*-12 diene system and a free carboxyl group (Kepler and Tove, 1967; Kepler *et al.*, 1970). If the initial isomerization involves the *cis*-12 double bond, then a *cis*-9, *trans*-11 conjugated diene is produced, whereas if the initial double bond isomerized is the *cis*-9, then *trans*-10, *cis*-12 conjugated diene is produced. Most rumen bacteria capable of carrying out this isomerization produce mainly RA from linoleic acid. However, Kim *et al.* (2002) recently demonstrated that the rumen bacterium, *Megasphaera elsdenii* YJ-4, produced predominately *trans*-10, *cis*-12 CLA and only a minor quantity of RA when incubated with linoleic acid. Nevertheless, the extent to which the various pathways of biohydrogenation are associated with specific enzymes and species of bacteria or reflect a general lack of specificity of the bacteria and their enzymes is not known.

It appears that the type of diet, rather than level of intake, is a major factor affecting biohydrogenation, and diet-induced changes in the rumen environment can shift the biohydrogenation pathways resulting in dramatic changes in the fatty acid intermediates. In addition, recent studies have established that the metabolism of radio-labelled oleic and eladic acids by mixed ruminal microorganisms results in extensive labelling of a wide range of *trans* octadecenoic fatty acids (*trans*-6 to *trans*-16) as well as of stearic acid (Mosley *et al.*, 2002; Proell *et al.*, 2002). In a few cases, the biological implications of the changes in the pathways of rumen biohydrogenation have been established and these will be discussed in later sections. A more comprehensive discussion of lipid metabolism in the rumen and its effects on the production of CLA and *trans* 18:1 isomers is provided in a recent review by Palmquist *et al.* (2005).

### 3.4.2. *cis*-9, *trans*-11 CLA (Rumenic Acid)

Initially, it was assumed that the RA in milk fat and body fat of ruminants originated from incomplete biohydrogenation in the rumen. This hypothesis was based on the fact that RA was the major CLA isomer in ruminant fat and the first intermediate in the major biohydrogenation pathway for linoleic acid (Figure 3.2). A close linear relationship was also observed between the levels of VA and RA in milk fat (Jiang *et al.*, 1996; Jahreis *et al.*, 1997; Lawless *et al.*, 1998; Griinari and Bauman, 1999), consistent with the concept that these two fatty acid intermediates had escaped complete biohydrogenation in the rumen and were subsequently absorbed from the digestive tract and used for milk fat synthesis. However, there were a number of inconsistencies with this idea. Firstly, the kinetics of rumen biohydrogenation are such that CLA represents only a transitory product, and VA is the major biohydrogenation intermediate that accumulates in the rumen (Keeney, 1970; Harfoot and Hazelwood, 1997). Secondly, nutrition studies demonstrated that increases in the milk fat content of CLA occurred when linseed oil and other dietary sources of linolenic acid were fed (e.g., Kelly *et al.*, 1998a; Dhiman *et al.*, 2000; Lock and Garnsworthy, 2002). As previously discussed, RA is not an intermediate in the biohydrogenation of linolenic acid, but the biohydrogenation of both linoleic and linolenic acids produces VA as an intermediate. Thirdly, the ratio of VA to RA is >50:1 in rumen fluid but only about 3:1 in milk fat. Based on these considerations, Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of the RA found in milk fat, with synthesis involving the enzyme  $\Delta^9$ -desaturase and VA as the substrate (see Figure 3.2). Previous investigations with  $\Delta^9$ -desaturase from rat liver established that while the preferred reaction was the conversion of stearic acid to oleic acid,

this enzyme could also desaturate positional isomers of *trans*-octadecenoic acids (Mahfouz *et al.*, 1980; Pollard *et al.*, 1980).

The first study to show directly that milk fat CLA could originate *via* endogenous synthesis was Griinari *et al.* (2000b); they infused 12.5 g/d of VA into the abomasum of dairy cows and observed a 31% increase in the concentration of RA in milk fat. This investigation clearly demonstrated the potential for endogenous synthesis, but additional studies were needed to determine its actual importance. To address this, two approaches have been used; one approach was to inhibit  $\Delta^9$ -desaturase directly, and this has involved the use of sterculic oil which contains two cyclopropene fatty acids, sterculic acid and malvalic acid, which are specific inhibitors of  $\Delta^9$ -desaturase (Phelps *et al.*, 1965; Bickerstaffe and Johnson, 1972; Jeffcoat and Pollard, 1977). To account for the fact that inhibition of  $\Delta^9$ -desaturase may not be complete, these investigations employed a correction factor based on the ratio in milk fat of *cis*-9 C<sub>14:1</sub> to C<sub>14:0</sub> (another product: substrate pair of  $\Delta^9$ -desaturase that is almost exclusively synthesized in the mammary gland). Griinari *et al.* (2000b), using this approach, estimated that 64% of the RA in milk fat was of endogenous origin in cows fed an alfalfa hay/corn grain-based diet. This represented the first direct demonstration that endogenous synthesis was the major source of RA in milk fat. Subsequent investigations using the same approach extended results to other dietary situations (total mixed diets with or without plant oils and pasture) and in all cases endogenous synthesis was the predominant source of the RA in milk fat (Corl *et al.*, 2001; Kay *et al.*, 2004). Results obtained with grazing cows are of special note because pasture is high in linolenic acid and endogenous synthesis accounted for >91% of the total RA in milk fat (Kay *et al.*, 2004).

The second approach to quantify the contribution of endogenous synthesis to milk fat CLA was indirect and involved a comparison of ruminal outflow with secretion in milk; rumen output of RA would represent the maximum proportion of RA secreted in milk fat and the remainder would have to be derived from endogenous synthesis. For this approach, representative samples of digesta were obtained and data for CLA content were combined with marker-derived estimates of flow rates of digesta. Lock and Garnsworthy (2002), who conducted the first such investigation, estimated rumen output of CLA in non-lactating cows and then extrapolated results to lactating cattle on the basis of feed intake. Their estimates indicated that endogenous synthesis accounted for over 80% of the RA in milk fat in cows fed a grass silage/concentrate diet supplemented with various plant oils. Comparable results were obtained in subsequent studies using this approach with cows fed corn-silage diets containing either a high or low level of forage (Piperova *et al.*, 2002), and grass silage/concentrate diets including fish oil supplements (Shingfield *et al.*, 2003). Recently, Palmquist *et al.* (2004)

used a mathematical modelling approach to quantify the importance of endogenous synthesis of CLA in adipose tissue of lambs, but it has not yet been applied to lactating cows.

Overall, investigators using different diets and experimental approaches have found similar results; the major source of RA in milk fat is endogenous synthesis (Figure 3.2). Thus, endogenous synthesis is the basis for *cis*-9, *trans*-11 being the predominant CLA isomer in milk fat and the relatively constant ratio between VA and RA observed in milk fat reflects the substrate: product relationship for  $\Delta^9$ -desaturase.

### 3.4.3. *trans*-7, *cis*-9 CLA

Yurawecz *et al.* (1998) were the first to identify the presence of *trans*-7, *cis*-9 CLA in milk fat and to do so they used combinations of silver nitrate-HPLC, GC-MS and Fourier transform infrared spectroscopy. This CLA isomer had not been detected previously because it co-eluted with RA in GC methods that were in routine use. Thus, concentrations of RA reported in the scientific literature typically include *trans*-7, *cis*-9 CLA as a component. Across studies, the level of *trans*-7, *cis*-9 CLA in milk fat has generally been on the order of 10% of RA and several-fold greater than any of the other CLA isomers (Sehat *et al.*, 1998; Yurawecz *et al.*, 1998; Bauman *et al.*, 2000; Corl *et al.*, 2002; Piperova *et al.*, 2002). Early investigations with  $\Delta^9$ -desaturase from rat liver had established that *trans*-7 18:1 could serve as a substrate for this enzyme (Mahfouz *et al.*, 1980; Pollard *et al.*, 1980). Furthermore, *trans*-7 18:1 is present in rumen outflow, albeit at low concentrations (Table 3.2), being produced as an intermediate in the biohydrogenation of oleic acid and 18-carbon PUFAs, as discussed earlier. Based on this, when Yurawecz *et al.* (1998) initially discovered *trans*-7, *cis*-9 CLA in milk fat, they speculated that it might originate from endogenous synthesis.

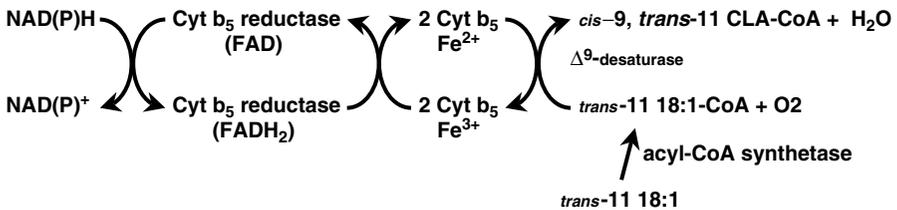
A number of the investigators who determined endogenous synthesis of RA also examined the source of *trans*-7, *cis*-9 CLA in milk fat. Corl *et al.* (2002) inhibited the activity of  $\Delta^9$ -desaturase in lactating dairy cows with sterculic oil as a source of cyclopropene fatty acids and with *trans*-10, *cis*-12 CLA, a specific inhibitor of both activity and gene expression for  $\Delta^9$ -desaturase (Lee *et al.*, 1998; Bretillon *et al.*, 1999; Baumgard *et al.*, 2000; Park *et al.*, 2000). Their data indicated that the *trans*-7, *cis*-9 CLA in milk fat was “derived almost exclusively from endogenous synthesis *via*  $\Delta^9$ -desaturase” (Corl *et al.*, 2002). Consistent with this, they also observed that there was no detectable *trans*-7, *cis*-9 CLA in rumen fluid. Piperova *et al.* (2002) used the indirect approach to calculate rumen outflow by combining duodenal content of *trans*-7, *cis*-9 CLA with an estimate of digesta flow. When estimates of rumen output of this CLA isomer were compared to that secreted in milk,

they concluded that “almost the entire [amount of] *trans*-7, *cis*-9 CLA” found in milk fat must be produced postruminally. Thus, results from these two very different approaches were in agreement that the source of *trans*-7, *cis*-9 CLA in milk fat was endogenous synthesis *via*  $\Delta^9$ -desaturase from ruminally produced *trans*-7 18:1.

### 3.4.4. The $\Delta^9$ -Desaturase Enzyme System

The predominance of endogenous synthesis as the source of RA and *trans*-7, *cis*-9 CLA in milk fat highlights the critical role of  $\Delta^9$ -desaturase in the biology of CLA. Although referred to as  $\Delta^9$ -desaturase in this review, this enzyme is also known as stearoyl-CoA desaturase (EC 1.14.19.1) in biochemistry texts because stearic acid is its most common substrate. The oxidative reaction catalyzed by  $\Delta^9$ -desaturase involves cytochrome  $b_5$ , NAD(P)-cytochrome  $b_5$  reductase and molecular oxygen (Figure 3.3). The CoA ester of VA is the substrate for the formation of RA, but the preferred substrates for  $\Delta^9$ -desaturase are stearoyl-CoA and palmitoyl-CoA, which are converted to oleoyl-CoA and palmitoleoyl-CoA, respectively (Ntambi, 1999). For ruminants, a substantial activity of  $\Delta^9$ -desaturase has been reported in mammary tissue (Bickerstaffe and Annison, 1970; Kinsella, 1972; McDonald and Kinsella, 1973; Wahle, 1974), adipose tissue (Wahle, 1974; Chang *et al.*, 1992; Cameron *et al.*, 1994; Barber *et al.*, 2000) and in intestinal epithelium (Bickerstaffe and Annison, 1969). In contrast to rodents, the ruminant liver has only negligible activity. Both bovine (Cooney and Headon, 1989; Chung *et al.*, 2000) and ovine (Ward *et al.*, 1998)  $\Delta^9$ -desaturase genes have been cloned and only one gene has been found. This is similar to humans, but differs from rodents where two isoforms of the gene have been identified in rats and three isoforms of the gene have been characterized in mice (Ntambi and Miyazaki, 2004).

Our understanding of the regulation of  $\Delta^9$ -desaturase in ruminants is limited, with current knowledge coming mainly from investigations on



**Figure 3.3.** The  $\Delta^9$ -desaturase enzyme system showing the conversion of vaccenic acid (*trans*-11 18:1) to rumenic acid (*cis*-9, *trans*-11 CLA).

rodents.  $\Delta^9$ -Desaturase has no known allosteric or feedback inhibition involving its substrates or products. However, it is regulated by dietary factors such as glucose and PUFAs, and by hormones such as insulin and glucagon (Ntambi and Miyazaki, 2004). The enzyme protein has a relatively short half-life ( $\sim 4$  h) and thus gene transcription is its major point of regulation (Ozols, 1997). Both PUFAs and *trans*-10, *cis*-12 CLA down-regulate gene expression, but RA has no effect (Lee *et al.*, 1998; Choi *et al.*, 2000; Ntambi and Miyazaki, 2004). Interestingly, the cyclopropene fatty acids in sterculic oil do not affect expression of the  $\Delta^9$ -desaturase gene or protein, but they directly inhibit the activity of the enzyme (Gomez *et al.*, 2003).

At a cellular level, regulation of  $\Delta^9$ -desaturase in mammary tissue appears to involve the sterol-response-element-binding-protein (SREBP) family of transcription factors (Peterson *et al.*, 2004). PUFAs inhibit the processing of SREBP-1 and may decrease the abundance of the precursor protein, leading to reduction in transcription of many genes in the lipogenic pathways, including  $\Delta^9$ -desaturase (Shimano, 2001; Horton *et al.*, 2002). Ward *et al.* (1998) reported high expression of  $\Delta^9$ -desaturase mRNA in adipose tissue and mammary gland of lactating sheep, and expression was decreased by 80% in adipose tissue of animals during pregnancy and lactation, a time when lipogenic activity is increased in mammary gland and decreased in adipose tissue (Bauman and Currie, 1980).

The relationship between substrate and product for  $\Delta^9$ -desaturase is reflected by the desaturase index, defined as  $[\text{RA} \div (\text{RA} + \text{VA})]$  (Kelsey *et al.*, 2003). The desaturase index in milk fat represents a proxy for  $\Delta^9$ -desaturase and a several-fold range is observed among individuals. This is discussed in Section 3.5.2, but provides a strong indication that there are genetic differences among individuals with respect to this enzyme.

### 3.4.5. Other CLA Isomers

In contrast to *cis*-9, *trans*-11 and *trans*-7, *cis*-9, the other isomers of CLA found in the milk fat of ruminants appear to originate exclusively from rumen output. This conclusion is based, in large part, on the fact that these minor *cis-trans*, *trans-cis*, *cis-cis*, and *trans-trans* isomers are detected in rumen fluid (Corl *et al.*, 2002) and duodenal fluid (Piperova *et al.*, 2002; Shingfield *et al.*, 2003), and estimates of digesta flow indicate that rumen output is more than adequate to account for the trace amounts secreted in milk fat (Piperova *et al.*, 2002; Shingfield *et al.*, 2003). Furthermore, there has been no demonstration that other mammalian desaturases act in a manner analogous to  $\Delta^9$ -desaturase to synthesize CLA endogenously from *trans* octadecenoic fatty acids. Thus, these CLA isomers found at trace levels

in milk fat are logically of rumen origin and represent intermediates formed in the biohydrogenation of PUFAs.

Information on the effect of diet on the production of minor isomers of CLA in the rumen and alterations in their content in milk fat is limited. Diet-induced changes in *trans*-10, *cis*-12 CLA have been best described, and its biological effects in the dairy cow will be discussed in Section 3.6.1. Griinari and Bauman (1999) presented a putative pathway for the biohydrogenation of linoleic acid where the initial isomerization involved the *cis*-9 double bond, thereby resulting in the production of *trans*-10, *cis*-12 CLA and *trans*-10 18:1 as intermediates. As discussed earlier, rumen bacteria have been identified that produce *trans*-10, *cis*-12 CLA when incubated with linoleic acid (Verhulst *et al.*, 1987; Kim *et al.*, 2002), and the addition of *trans*-10, *cis*-12 CLA to the rumen results in the increased formation of *trans*-10 18:1 (Loor and Herbein, 2001).

Diet has also been shown to influence rumen output and milk fat content of other minor CLA isomers, although they always remain a small portion of total CLA in milk fat. For example, dietary supplements rich in linolenic acid increased the relative proportions of *trans*-11, *cis*-13 CLA, *trans*-11, *trans*-13 CLA, *cis*-12, *trans*-14 CLA, and *trans*-12, *trans*-14 CLA (Griinari *et al.*, 2000a; Griinari and Shingfield, 2002). Ruminant output of *trans-trans* CLA isomers with double bonds at positions 9, 11 and 10, 12 was enhanced when diets contained high amounts of concentrates (Piperova *et al.*, 2002) or were supplemented with fish oil (Shingfield *et al.*, 2003). Recently, Kraft *et al.* (2003) reported that *trans*-11, *cis*-13 CLA represented 2 to 8% of total CLA in milk fat from cows grazing in the Alps. Kramer *et al.* (2004) verified this when they found relatively high concentrations of this isomer in a cheese sample produced from the milk of Alpine cows and *trans*-11, *cis*-13 CLA was also observed at significant concentrations in a sample of Yak milk fat (Cruz-Hernandez *et al.*, 2004). Kraft *et al.* (2003) suggested that the *trans*-11, *cis*-13 CLA might be produced *via* rumen biohydrogenation of linolenic acid based on the lipid content of Alpine pasture, but this has not been established directly. Overall, most investigations of the effects of diet on CLA have not used the detailed analytical methods required to resolve the full range of minor CLA isomers. Thus, more completely identifying rumen biohydrogenation pathways and establishing their relationship to specific rumen bacteria and diets are important areas for future research.

### 3.5. Modification of CLA Content in Milk Fat

The discovery of health benefits of CLA and a recognition of the potential of RA as a functional food component in dairy products has stimulated research to identify factors that affect the CLA content of milk fat. These

efforts have focused on enhancing the CLA content per unit of fat and centered on RA as the predominant CLA isomer. From the preceding discussion on the origin of RA (Section 3.4.2), there are four possibilities to consider: (i) increase the 18-carbon PUFA precursors in the diet (linoleic and linolenic acids); (ii) maintain rumen biohydrogenation pathways that result in the production of VA as an intermediate; (iii) inhibit the final step in the biohydrogenation of 18-carbon PUFAs so that VA accumulates; and (iv) increase  $\Delta^9$ -desaturase and the desaturation of VA to RA in the mammary gland. In the following sections, we will discuss results of investigations designed to establish dietary and nutritional conditions that maximize rumen outflow of VA and RA, optimize the amount and activity of  $\Delta^9$ -desaturase in mammary tissue, and identify the physiological basis for the large differences among individuals in terms of the production of CLA. Obviously, before CLA-enriched foods are widely marketed, effects on quality and consumer acceptability of the dairy products also need to be examined, and the limited research on this will be summarized also.

### 3.5.1. Dietary and Nutritional Effects

Numerous studies have shown that diet is the most significant factor affecting the CLA content of milk fat, and its concentration can be increased several-fold by dietary means (see reviews by Chilliard *et al.*, 2000; 2001; Bauman *et al.*, 2001; Stanton *et al.*, 2003; Lock and Bauman, 2004). As cited above, one key to increasing milk CLA is to increase the dietary intake of 18-carbon PUFAs, thereby providing more substrate for rumen biohydrogenation. The dietary supply of linoleic and linolenic acids is most easily increased by the addition of plant oils rich in these fatty acids, and a number of plant oils have been investigated and shown to be effective in increasing the level of CLA in milk fat. For example, dietary supplements of soybean, sunflower, rapeseed or linseed oils have been used successfully to increase the level of CLA in milk fat (Kelly *et al.*, 1998a; Dhiman *et al.*, 2000; Chouinard *et al.*, 2001; Lock and Garnsworthy, 2002).

The slow release of PUFAs in the rumen typically creates favourable conditions for the accumulation of *trans*-18:1 fatty acids, thereby increasing rumen output of VA (Bauman *et al.*, 2001). In this regard, the coat of oil seeds offers some protection against rumen biohydrogenation and thus, the use of different oil seeds and processing techniques have been investigated also; correspondingly, a range of oilseeds containing both linoleic and linolenic acids have been shown to be affective in increasing the CLA content of milk fat (e.g., Stanton *et al.*, 1997; Dhiman *et al.*, 2000; AbuGhazaleh *et al.*, 2001; Chouinard *et al.*, 2001). In general, oil seeds that are rich in 18-carbon PUFAs

and processed so that the oil is accessible to the bacteria involved in biohydrogenation result in greater increases in milk CLA compared with whole oil seeds, but are not as efficient as using the pure oil (Lock and Bauman, 2004). The use of calcium salts of fatty acids derived from plant oils has also been investigated because of the partial protection that the calcium-fatty acid complex offers from rumen biohydrogenation. Chouinard *et al.* (2001) fed calcium salts of fatty acids derived from rape, soybean and linseed oils; all three increased the CLA content of milk fat, with the largest increases occurring for those containing the greatest amounts of linoleic and linolenic acids (soybean and linseed, respectively).

The amount of 18-carbon PUFAs that can be added to the diets of dairy cows is limited due to the adverse effects these PUFAs can have on the metabolism of rumen bacteria, thereby impairing rumen fermentation and animal performance (Jenkins, 1993). Thus, dairy cattle diets are generally restricted to less than 7% total lipid, and this provides an upper limit to the use of lipid supplements. Oilseeds and chemical protection of oils offer some benefit as they often allow for greater amounts of the oil to be fed before negative effects on microbial growth and metabolism are realized. When a high level of oil is added, up to 10-fold increases in the CLA content of milk fat are observed, but because of negative effects on rumen bacteria discussed above, these levels are often transient and decline within a few weeks to stabilize at ~4-fold to 5-fold increases (e.g., Bauman *et al.*, 2000). In addition, there is often a fine line between supplying additional lipid supplements to increase milk fat CLA content and causing changes in the rumen environment; for example, under some conditions the rumen environment may be modified to produce more *trans*-10 18:1 and *trans*-10, *cis*-12 CLA as intermediates and this results in a dramatic reduction in milk fat synthesis (see Section 3.6.1).

Another means through which dietary and nutritional factors can increase the CLA content of milk fat is by inhibiting the terminal step in biohydrogenation (Figure 3.2). This typically occurs either directly or indirectly *via* changes in the rumen environment; the net result is an accumulation of VA, thereby increasing the rumen outflow of this precursor for the endogenous synthesis of CLA. A limited number of bacterial species have been shown to carry out the final biohydrogenation step and, presumably, changes in the rumen environment lead to a reduction in these species and/or a reduction in their capacity to reduce VA to stearic acid. Several dietary situations also have these effects and they include alterations in the forage: concentrate ratio, dietary supplements of fish oil and restricted feeding (see Bauman *et al.*, 2001). The most consistently effective of these is the use of fish oils. Fish oils themselves provide very little 18-carbon PUFAs precursors to allow for increased rumen VA output, indicating that this increase occurs

through an inhibition of the biohydrogenation of VA; indeed, C22:6 n-3 (docosahexaenoic acid, DHA), a major n-3 fatty acid in fish oil, has been shown to promote the accumulation of VA in mixed ruminal cultures when incubated with linoleic acid (AbuGhazaleh and Jenkins, 2004). Both linoleic and linolenic acids are plentiful in forages and concentrates which provide sufficient 18-carbon PUFA precursors. A range of fish and marine oils have been used with success, and similar to the supply of 18-carbon PUFAs discussed above, both lipid supplements and fish by-products (fish meal) have been shown to be effective (e.g., Offer *et al.*, 1999; Donovan *et al.*, 2000; AbuGhazaleh *et al.*, 2001; Shingfield *et al.*, 2003). Marine algae also contain long chain PUFAs and have also been effective (Franklin *et al.*, 1999).

The most effective dietary treatments for increasing the CLA content of milk fat are those that both increase the supply of 18-carbon PUFAs and modify the rumen environment. The most widely studied of these is the use of fresh pasture, with numerous studies indicating that fresh pasture results in a 2-fold to 3-fold increase in the CLA content of milk fat (e.g., Stanton *et al.*, 1997; Kelly *et al.*, 1998b; Dhiman *et al.*, 1999). The degree of response, however, decreases as the pasture matures and the proportion in the diet decreases. Correspondingly, seasonal effects on milk CLA content have been reported, with the trend that the content is greatest when fresh pasture is plentiful, and decreases throughout the growing season (Riel, 1963; Banni *et al.*, 1996; Auldust *et al.*, 2002; Lock and Garnsworthy, 2003). These results cannot be explained fully in terms of the fatty acid composition and supply of PUFAs that grass provides; therefore, there must be additional factors or components of grass that promote the production of VA in the rumen, and these lessen in effect as the pasture matures (Lock and Bauman, 2004). Presumably, these factors inhibit the conversion of VA to stearic acid, as discussed previously. The effect of different farming systems has also been investigated, with systems differentiated by the amount and type of forage typically fed to cows. In general, production systems with the greatest proportion of fresh forage in the diet give the highest level of CLA in milk fat. For example, Jahreis *et al.* (1997) reported that cows grazed during the summer months had a higher level of CLA in milk than cows housed all-year round and fed conserved forage.

Although the use of fresh pasture has striking effects on enhancing the CLA content of milk fat, a similar increase is possible using standard dietary ingredients such as plant oils/oilseeds and fish oil/fish meal supplements. Further, there is some indication that dietary regimes involving a combination of supplements can have an additive effect on increasing the level of CLA in milk; for example Whitlock *et al.* (2002) observed higher levels with a combination of plant oil and fish oil than when either was fed alone. In all of the dietary situations designed to enhance the level of CLA in milk fat, it

is vital that the normal VA pathway of biohydrogenation is maintained. If shifts in biohydrogenation occur, then the pattern of *trans* fatty acids changes and there will be a reduction in the rumen output of VA, and as a consequence a reduction in the level of CLA in milk fat. This shift in the pathways of biohydrogenation is also associated with an increased risk of depression of milk fat synthesis (see Section 3.6.1).

### 3.5.2. Physiological Factors

Physiological factors also have an impact on the content of CLA in milk fat. Surveys have shown an 8-fold range in the milk fat content of CLA among herds (Riel, 1963; Kelly and Bauman, 1996) and these differences in large part reflect diet and nutritional effects as discussed above. However, substantial differences are observed among cows within a herd consuming the same diet. Investigations involving diets ranging from corn-based total mixed rations to pasture have all shown a 2-fold to 3-fold range in the milk fat content of CLA among individual cows (e.g., Kelly *et al.*, 1998a, b; Lawless *et al.*, 1998; Lock and Garnsworthy, 2002; 2003; Peterson *et al.*, 2002b). Thus, across diets that result in substantial differences in the average milk fat content of CLA, a similar 2-fold to 3-fold range is observed among cows consuming the same diet. This variation would in large part be related to individual differences in, (1) rumen output of VA and to a lesser extent CLA, and (2) the amount and activity of  $\Delta^9$ -desaturase.

The final method to enhance the level of CLA in milk is to increase endogenous synthesis and this probably explains the variation among cows in a herd fed the same diet. Undoubtedly, the variation in  $\Delta^9$ -desaturase among individuals has a genetic basis (Bauman *et al.*, 2003), but this has not been examined directly. However, an indirect evaluation is possible because milk fat contains four major fatty acid pairs that represent a product/substrate relationship for  $\Delta^9$ -desaturase, myristoleic/myristic acid, palmitoleic/palmitic acid, oleic/stearic acid and RA/VA. Ratios for these pairs of fatty acids, referred to as a desaturase index, represent a proxy for  $\Delta^9$ -desaturase activity. In the largest study to examine this hypothesis, Kelsey *et al.* (2003) found that the variation in milk fat content of RA and the desaturase index was about 3-fold among individuals consuming the same diet. Other investigators have also observed a 2-fold to 3-fold range in desaturase index among cows in the same herd (Lock and Garnsworthy, 2002; 2003; Peterson *et al.*, 2002b). Peterson *et al.* (2002b) also demonstrated a consistency in the individual hierarchy in desaturase index over time when cows were fed the same diet and a consistency in the individual hierarchy when cows were switched between diets. Presumably, this variation reflects individual differences in the activity of  $\Delta^9$ -desaturase involving the regulation of gene expression, primary

or tertiary structure of the enzyme due to gene polymorphisms, post-translational modifications, or other factors affecting the interaction between the enzyme and the substrate or product.

Several specific physiological factors have been examined for effects on the level of CLA in milk fat, but because of the large impact of diet and the wide range among individuals, it is important that these comparisons involve a reasonable number of cows fed a common diet. These conditions were met in the studies by Kelsey *et al.* (2003) and Lock *et al.* (2005a), and both found that the CLA content of milk fat and the desaturase index had no relationship to parity or stage of lactation (days in milk). Likewise, they observed that the milk fat content of CLA and desaturase index also had no relationship to milk yield, milk fat percentage or yield of milk fat (Kelsey *et al.*, 2003; Lock *et al.*, 2005a). The investigation by Kelsey *et al.* (2003) involved over 200 cows fed the same diet and showed no difference between Holstein and Brown Swiss breeds. In contrast, several studies have reported breed differences in the CLA content of milk fat (Lawless *et al.*, 1999; White *et al.*, 2001; Whitlock *et al.*, 2002), which may reflect differences in desaturase index among breeds. However, these studies often involved very few animals or were confounded by diet, or both. Using a larger data set, DePeters *et al.* (1995) reported breed differences in the desaturase index in the milk fat of dairy cows, consistent with the suggestion that the activity of  $\Delta^9$ -desaturase is higher in Holstein than in Jersey mammary tissue (Beaulieu and Palmquist, 1995). However, if breed differences exist they would appear to be minor compared with the magnitude of dietary effects and variation among cows in terms of both the CLA content of milk fat and desaturase index.

Increasing  $\Delta^9$ -desaturase activity would not only impact on the level of CLA in milk fat, but would also increase other unsaturated fatty acids that are products of this enzyme. As a consequence of these changes, the saturated:unsaturated content of milk fat would be altered resulting in an improvement in the “human health” characteristics of milk fat. Thus, establishing the heritability of individual differences in the desaturase index and the extent to which this could be used in genetic selection programmes is of interest. This potential to improve the fatty acid composition of milk fat is also the basis for recent work to produce transgenic goats that have greater expression of  $\Delta^9$ -desaturase in the mammary gland (Reh *et al.*, 2004).

### 3.5.3. Manufacturing and Product Quality Considerations

Consumer surveys indicate an interest in dairy products that are enriched in CLA (Ramaswamy *et al.*, 2001b). As outlined in preceding sections, the level of CLA in milk fat can be enhanced several-fold naturally by diet formulation and selection of individual cows with elevated milk fat CLA.

But central to marketing and consumer acceptance of CLA-enriched foods is a consideration of the effects of processing and storage, and the final sensory characteristics of CLA-enriched products. Many dairy products undergo a microbial fermentation during processing and the effects of these on the CLA content have been of special interest. Several studies have investigated this and found that food processing and manufacturing have little or no effect on CLA content (Shantha *et al.*, 1992, 1995; Werner *et al.*, 1992; Jiang *et al.*, 1997; Lin *et al.*, 1999; Gnädig *et al.*, 2004). As emphasized in the review by Parodi (2003), any changes in the CLA content related to processing or to storage are minimal when compared to the variations associated with diet formulations and differences between individual cows. Thus, the final concentration of CLA in dairy products is, in large part, related to the CLA concentration in the raw milk fat and the fat content of the final product.

Consumer acceptability of CLA-enriched dairy products is also dependent on their taste and organoleptic properties. Off-flavours due to fatty acid oxidation are of prime concern because diet formulation methods used to enhance milk fat with respect to CLA generally cause an increase in the proportion of unsaturated fatty acids in the milk fat (Lock and Bauman, 2004). Reports on sensory characteristics and quality of naturally-enriched dairy products, typically having a 2-fold to 3-fold increase in milk fat CLA, have generally indicated no differences from unenriched dairy products (Ramaswamy *et al.*, 2001a, b; Baer *et al.*, 2001; Avramis *et al.*, 2003; Gonzalez *et al.*, 2003). An exception is Lacasse *et al.* (2002) who found that 2.7%-fat milk from cows fed either protected (3% of dry matter) or unprotected fish oil (3.7% of dry matter) scored significantly lower in flavor and taste. However, the levels of fish oil used in this study were significantly greater than used by others.

Lynch *et al.* (2005) compared the flavor, organoleptic and storage characteristics of standard 2%-fat milk with 2%-fat milk that had an approximately 10-fold higher level of CLA. The naturally enhanced milk (the level of CLA and VA was 47 and 121 mg/g fatty acids, respectively) was produced through individual selection and nutritional management of the cows. Initial evaluation of the milk and evaluation over a 14-day post-pasteurization period indicated no flavor differences as determined by triangle taste tests. Similarly, sensory results indicated no differences in susceptibility to the development of oxidized off-flavors between the control and CLA-enhanced milks, even when milk was stored under light (Lynch *et al.*, 2005). Thus, flavor and consumer acceptability were maintained in a dairy product with substantially enhanced levels of CLA and VA.

The research discussed above involved dairy products that were naturally enriched with CLA through formulation of diets known to increase

the level of CLA in milk fat and selection of individual cows with a higher level of CLA in their milk fat. Campbell *et al.* (2003) used an alternative approach involving fortification of milk fat with synthetic CLA during the manufacturing process. They added 1 or 2% CLA-containing triglycerides to skim milk together with vitamin E and rosemary extract to retard lipid oxidation. Descriptive sensory analysis revealed that the fortified milk had a “grassy/vegetable oil” flavor and consumer acceptability scores were low, although acceptability was improved when a chocolate flavor was added.

Overall, results to date indicate that manufacturing and quality characteristics were normal for dairy products naturally enriched with CLA and consumer acceptability was comparable to unenriched dairy products. However, the single study examining fortification of skim milk with synthetic CLA during the manufacturing process had poor consumer acceptability.

### 3.6. Biological Effects of CLA Isomers

A broad overview of the biological effects of CLA is presented elsewhere in this volume (Chapter 17), so the emphasis in the following section will be two-fold. Firstly, the biology of *trans*-10, *cis*-12 CLA in the dairy cow will be summarized because under certain dietary conditions, production of this isomer in the rumen can profoundly affect milk fat synthesis. Secondly, the biological effects of RA when supplied as a natural component of the diet will be reviewed because this CLA isomer represents a functional component of milk fat that has potential health benefits. Although other CLA isomers are present in milk fat, they are present at concentrations much too low to have a significant effect.

#### 3.6.1. *trans*-10, *cis*-12 CLA and Lipid Metabolism

##### 3.6.1.1. Inhibition of Milk Fat Synthesis

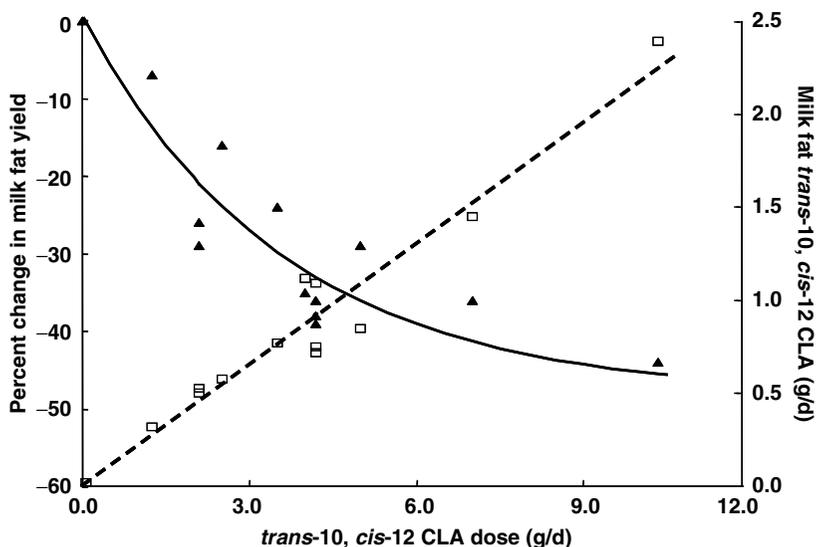
Investigations in which the transfer of CLA to milk fat in dairy cows was examined showed that supplementation of mixed isomers of CLA resulted in a dramatic reduction in milk fat secretion (Loor and Herbein, 1998; Chouinard *et al.*, 1999a, b). Decreases of up to 50% in milk fat yield occurred and the effects were reversed when supplementation was terminated. Furthermore, effects were specific for milk fat with the yield of milk and other milk components being relatively unaffected. Initial investigations were of short duration (<7 days) and the CLA supplement was infused abomasally as a convenient experimental method to avoid possible alterations during rumen fermentation. However, subsequent long-term studies (20 weeks) demonstrated that the reduction in milk fat synthesis was

maintained when a rumen-protected formulation of CLA was used (Perfield *et al.*, 2002; Bernal-Santos *et al.*, 2003).

Early investigations utilized CLA supplements that were composed of a mixture of, generally, four or more isomers. Baumgard *et al.* (2000) reported the first evidence of the differential effect of specific CLA isomers on milk fat synthesis; they demonstrated that abomasal infusion of *trans*-10, *cis*-12 CLA resulted in an immediate decrease in milk fat synthesis whereas RA had no effect. Recently, additional CLA isomers have been examined *via* abomasal infusion and these have included *trans*-8, *cis*-10 CLA, *cis*-11, *trans*-13 CLA, and *trans*-10, *trans*-12 CLA (Perfield *et al.*, 2004a, c). Although all of these isomers were taken up by the mammary gland and incorporated into milk fat, none affected the rate of milk fat synthesis. Thus, *trans*-10, *cis*-12 CLA is the only CLA isomer that has been shown to reduce milk fat synthesis. The initial step in the metabolism of linoleic and linolenic acids to form eicosanoids is catalyzed by  $\Delta^6$ -desaturase. The metabolite formed by the action of  $\Delta^6$ -desaturase on *trans*-10, *cis*-12 CLA is *cis*-6, *trans*-10, *cis*-12 18:3. Investigations of this fatty acid, as well as *cis*-6, *trans*-8, *cis*-12 18:3, have established that neither of these conjugated trienoic 18:3 fatty acids affect milk fat synthesis or any other lactational variable (Sæbø *et al.*, 2005).

Relationships between *trans*-10, *cis*-12 CLA and milk fat synthesis have been examined. There is a curvilinear relationship between the reduction in milk fat yield and the abomasal infusion dose of *trans*-10, *cis*-12 CLA (Figure 3.4). *Trans*-10, *cis*-12 CLA is a very potent inhibitor of milk fat synthesis in dairy cows; a dose of 2.0 g/d (<0.01% of dry matter intake) reduced milk fat synthesis by 20%. *Trans*-10, *cis*-12 CLA is also incorporated into milk fat and in this case the relationship is linear (Figure 3.4); a summary of seven studies showed that the transfer efficiency of abomasally-infused *trans*-10, *cis*-12 CLA into milk fat averaged 22% (de Veth *et al.*, 2004). The linear relationship in transfer to milk fat is remarkable when one considers that the yield of milk fat is simultaneously decreased as the abomasal dose of *trans*-10, *cis*-12 CLA is increased. This suggests that the mechanisms which coordinate the CLA-induced decrease in the use of preformed fatty acids for milk fat synthesis have a less pronounced effect on the mammary uptake and incorporation of *trans*-10, *cis*-12 CLA into milk fat, but the basis for this difference is unknown.

Initial studies on CLA showed that the reduction in milk fat secretion reflected decreases in fatty acid levels of all chain-length, but effects were most pronounced for those synthesized *de novo* (Loor and Herbein, 1998; Chouinard *et al.*, 1999a,b). As investigations focused on *trans*-10, *cis*-12 CLA and expanded to include a range of doses, it was discovered that at lower doses, the reduction in milk fat was distributed more uniformly among the fatty acids synthesized *de novo* (short-chain and medium-chain length)



**Figure 3.4.** Relationships between dose of *trans*-10, *cis*-12 CLA infused into the abomasum and (i) change in milk fat yield (▲;  $y = -48.26 + 49.03 \exp^{-0.2782x}$ ;  $R^2 = 0.86$ ), and (ii) secretion of *trans*-10, *cis*-12 CLA into milk fat (□;  $y = 0.2175x + 0.0111$ ;  $R^2 = 0.94$ ). Adapted from a summary by de Veth *et al.* (2004) using data from Baumgard *et al.* (2000, 2001, 2002), Peterson *et al.* (2002a), Sæbø *et al.* (2005), de Veth *et al.* (2004) and Perfield *et al.* (2004c).

and the longer-chain fatty acids taken up from the blood (Baumgard *et al.*, 2001; Peterson *et al.*, 2002a). Likewise, an inhibition of  $\Delta^9$ -desaturase which resulted in a marked shift in the fatty acid composition of milk fat was observed only at doses of *trans*-10, *cis*-12 CLA where milk fat production was reduced by >20%. At lower doses of *trans*-10, *cis*-12 CLA, the ratio of fatty acids representing product/substrate for  $\Delta^9$ -desaturase was unaffected (Baumgard *et al.*, 2001; Peterson *et al.*, 2002a; de Veth *et al.*, 2004).

The changes observed in the fatty acid composition of milk in CLA-supplemented cows suggest that many of the processes involved in milk fat synthesis must be affected. Baumgard *et al.* (2002) conducted the first investigation of this by quantifying the abundance of mRNA for several lipogenic enzymes in mammary tissue obtained 5 days after treatment with *trans*-10, *cis*-12 CLA. They found that the 48% reduction in milk fat yield corresponded to a reduction of similar magnitude in the abundance of mRNA for genes that encoded for enzymes involved in the uptake and transport of circulating fatty acids (lipoprotein lipase and fatty acid-binding protein), *de novo* fatty acid synthesis (acetyl CoA carboxylase and fatty acid synthase), desaturation of fatty acids ( $\Delta^9$ -desaturase), and triglyceride

synthesis (glycerol phosphate acyltransferase and acylglycerol phosphate acyltransferase). Subsequent work using a bovine mammary epithelial cell line has given similar results when cells were incubated with *trans*-10, *cis*-12 CLA (Peterson *et al.*, 2004).

The biochemical responses described above support the hypothesis that the reduction in the production of milk fat involves a coordinated regulation of key lipogenic enzymes in the mammary gland, and logical candidates as a central regulator of lipid synthesis are the sterol-response-element-binding-proteins (SREBP). The role of SREBP in the regulation of lipid metabolism has been characterized elegantly in rodents where promoters for sterol response elements have been identified in genes for key enzymes in the pathways of fatty acid synthesis and metabolism (see reviews by Shimano, 2001; Horton *et al.*, 2002). Recently, Peterson *et al.* (2004) found that bovine mammary epithelial cells also contain SREBP. They demonstrated further that *trans*-10, *cis*-12 CLA reduced lipid synthesis in these cells through inhibition of the proteolytic activation of SREBP-1 and subsequent reduction in translational activation of lipogenic genes. Thus, the mechanism whereby *trans*-10, *cis*-12 CLA affects milk fat synthesis appears to involve alterations in the activation of this transcription factor.

### 3.6.1.2. Relationship to Diet-Induced Milk Fat Depression

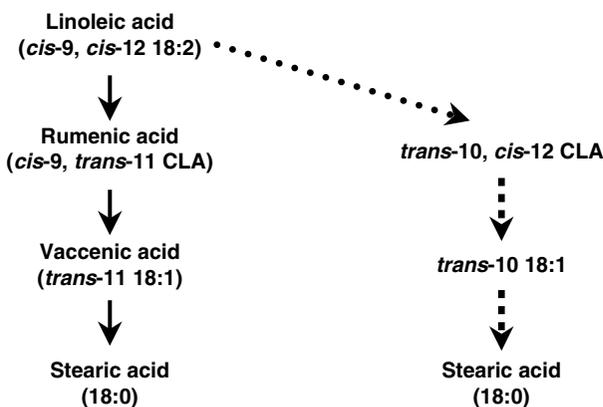
Under particular dietary situations, a reduction in the content and yield of milk fat occurs in dairy cows. This has commonly been referred to as milk fat depression (MFD) and recent investigations indicate that this metabolic syndrome is related, at least in part, to effects of specific CLA isomers on rates of milk fat synthesis. First described over a century ago, MFD has most often been observed with diets that are low in roughage and high in starch, diets containing plant or fish oil supplements and diets where effective fiber is reduced by processing the forage (e.g., grinding or pelleting). Effects are specific for milk fat and decreases of up to 50% have been observed.

Diet-induced MFD has been the subject of extensive research, especially over the last 50 years (see reviews by Davis and Brown, 1970; Palmquist *et al.*, 1993; Bauman and Griinari, 2001). Many theories have been advanced to explain diet-induced MFD, however, most have been found inadequate to explain the cause and mechanism of this phenomenon (Doreau *et al.*, 1997a; Bauman and Griinari, 2001, 2003). A shift in rumen fermentation is clearly involved and the occurrence corresponds to a marked increase in the *trans* 18:1 content of milk fat (Davis *et al.*, 1970; Griinari *et al.*, 1998). While VA is generally the principal *trans* 18:1 isomer in milk fat, a key development was the discovery by Griinari *et al.* (1998) that the change

with diet-induced MFD specifically involved an increase in the *trans*-10 18:1 isomer. Subsequently, this was verified for other dietary conditions (Piperova *et al.*, 2000; Offer *et al.*, 2001; Peterson *et al.*, 2003), and it established that diet-induced MFD involved a shift in the rumen pathways of biohydrogenation, as indicated in Figure 3.5.

Bauman and Griinari (2001) recognized the central role of rumen biohydrogenation in MFD and proposed that “under certain dietary conditions, the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates which are potent inhibitors of milk fat synthesis.” This is referred to as the “biohydrogenation theory” and results have demonstrated that diet-induced MFD is generally correlated with the level of *trans*-10, *cis*-12 CLA in milk fat (Bauman and Griinari, 2001; Peterson *et al.*, 2003; Piperova *et al.*, 2004). Further, Bauman and Griinari (2001) suggested that additional unique biohydrogenation intermediates that inhibit fat synthesis may be produced under dietary conditions causing MFD and recent work has offered further support for this idea (Perfield *et al.*, 2002; Peterson *et al.*, 2003; Piperova *et al.*, 2004). The level of *trans*-10 18:1 in milk fat is also highly correlated with the onset of diet-induced MFD, but to date there have been no investigations establishing a direct effect of this fatty acid (see discussion by Bauman and Griinari, 2003). Thus, at this time, the *trans*-10, *cis*-12 CLA isomer is the only rumen biohydrogenation intermediate shown to inhibit the synthesis of milk fat.

Recent investigations of the mechanism of diet-induced MFD indicate that it involves a coordinated reduction in the abundance of mRNA for key



**Figure 3.5.** Generalized scheme of ruminal biohydrogenation of linoleic acid under normal conditions (solid line) and during diet-induced milk fat depression (dotted line). Adapted from Griinari and Bauman (1999).

enzymes involved in the pathways of milk fat synthesis (Piperova *et al.*, 2000; Ahnadi *et al.*, 2002; Peterson *et al.*, 2003). Thus, mechanisms appear to be identical to those discussed earlier to explain the reduction in the production of milk fat observed with dietary supplementation with *trans*-10, *cis*-12 CLA. Overall, diet-induced MFD represents a natural situation where the production of *trans*-10, *cis*-12 CLA, and probably other unique biohydrogenation intermediates in the rumen, results in a decrease in mammary synthesis of fatty acids and a reduction in milk fat secretion. As knowledge of the biology of CLA increases, comparisons with the physiology of diet-induced MFD will continue to be of interest.

### 3.6.1.3. Use as a Management Tool

Dietary supplementation with CLA to reduce milk fat yield has potential use as a management tool in milk production. Milk fat is the major “cost” of milk synthesis accounting for over one-half of the energy needed for milk synthesis; consequently, a reduction in milk fat output will result in a sparing of energy that can be used for other purposes. Commercial situations where this could have application include markets where production is regulated by a quota system based on milk fat, and nutritional situations where cows cannot consume sufficient energy to meet their requirements. Examples of the latter include the onset of lactation and the early lactation period, and under adverse environmental conditions such as heat stress or weather-related feed shortages (see Griinari and Bauman, 2003).

Commercial application of *trans*-10, *cis*-12 CLA as a management tool requires a CLA formulation that must have two characteristics; it must offer protection of the CLA from alterations by rumen bacteria and the CLA must subsequently become available for absorption in the small intestine. The majority of protection methods are pH-dependent and take advantage of the transition occurring between rumen pH (~5.8 to 6.7) and pH of the abomasum (~2 to 4). To date, the majority of research on rumen-protected CLA has used supplements consisting of calcium salts of free fatty acids. Perfield *et al.* (2002) used this formulation in the first long-term investigation using cows in late lactation; they observed that the reduction in the production of milk fat (23% decrease) was maintained over the 20 week treatment period, whereas yields of milk and other milk components, maintenance of pregnancy and cow well-being were unaffected. A consistent reduction in the level of milk fat has also been observed in subsequent studies using calcium salts of CLA over treatment periods ranging from 3 to 20 weeks involving primiparous and multiparous cows at different stages of lactation and under different dietary and management practices (Giesy *et al.*, 2002;

Bernal-Santos *et al.*, 2003; Moore *et al.*, 2004; Piperova *et al.*, 2004; Selberg *et al.*, 2004).

The preparation of dietary supplements containing CLA using other methods of rumen-protection has been investigated less extensively compared to calcium salts of CLA, but have included formulations where the protection was by treatment with formaldehyde, the formation of amide bonds and lipid-encapsulation (de Veth *et al.*, 2003; Perfield *et al.*, 2004b). The transfer of *trans*-10, *cis*-12 CLA to milk fat offers a convenient method to evaluate the effectiveness of rumen protection methods. While all methods resulted in a reduction in the production of milk fat, transfer efficiency of *trans*-10, *cis*-12 CLA from rumen-protected supplements was much lower than the ~22% transfer efficiency reported for investigations involving abomasal infusions (de Veth *et al.*, 2004). Thus, the CLA in these formulations is protected only partially from rumen biohydrogenation and there is some indication that the rumen metabolism of a portion of the dietary supplement of CLA may result in the production of other fatty acids that are also able to inhibit milk fat synthesis (Perfield *et al.*, 2002; Piperova *et al.*, 2004). Additional aspects of the potential application of dietary supplements of *trans*-10, *cis*-12 CLA as a management tool are discussed by Griinari and Bauman (2003).

## 3.6.2. Rumenic Acid and Human Health

### 3.6.2.1. Cancer

Since the original discovers of the antimutagen properties of CLA (Pariza *et al.*, 1979; Ha *et al.*, 1987), its anticarcinogenic effects have received widespread interest. There are biomedical models for most types of cancer and many of these have been used to investigate the role of CLA as an anticarcinogen (see reviews by Scimeca, 1999; Belury, 2002; Banni *et al.*, 2003; Parodi, 2004). These include the use of human cancer cell lines, transplanted cell lines and *in situ* organ site carcinogenesis models. The latter are of particular value in cancer investigations and dietary supplements of CLA have been shown to be effective in inhibiting chemically-induced skin papillomas, fore-stomach neoplasia, and preneoplastic lesions and tumors in the colon and mammary gland (see Parodi, 2004; Bauman *et al.*, 2005). The majority of studies have used a mixture of CLA isomers produced synthetically from vegetable oil, typically containing 2 or 4 predominant isomers; the 2-isomer mix contains almost equal proportions of RA and *trans*-10, *cis*-12 CLA whereas the 4-isomer mixture also includes the *trans*-8, *cis*-10 and *cis*-11, *trans*-13 isomers. The anticarcinogenic effect of CLA is particularly impressive in studies on chemically-induced mammary cancer; dietary intake of CLA gives a dose-dependent reduction in the incidence and number of tumors (Ip *et al.*, 1991) and is independent of the type or level of fat in the

diet (Ip *et al.*, 1991, 1994). Most impressive is the fact that feeding CLA during the peripubertal period provided protection against mammary tumor development even when the carcinogen was administered at a later time (Thompson *et al.*, 1997). Conversely, when rats received no CLA supplementation until they were older and had mature mammary glands, the protective effect was achieved only when CLA was fed continuously during the tumor promotion period following administration of the carcinogen (Ip *et al.*, 1995).

The use of a functional food approach would have many advantages as a strategy to prevent cancer. Since CLA is found predominately in dairy fats in human diets, a series of studies have used the rat prepubertal mammary cancer model to investigate the anticarcinogenic potential of CLA when supplied as a naturally-enriched butter that was produced using dietary regimes described in Section 3.5. As discussed in Section 3.4, the majority of the RA in milk fat is synthesized endogenously from VA, and, as a consequence, the levels of VA and CLA in milk fat generally approximate a 3:1 ratio and change in concert (Bauman *et al.*, 2003; Palmquist *et al.*, 2005). Thus, the enriched butter is higher in both RA and VA. The initial investigation established that RA was an effective anticarcinogen when it was supplied as a dietary food in a natural form (esterified in triglycerides; Table 3.3; Ip *et al.*, 1999). Importantly, tissue concentrations of RA were greater in rats fed the VA/RA-enriched butter than for rats fed a comparable amount of chemically-synthesized RA, suggesting the possibility of endogenous synthesis from VA. As discussed in Section 3.4.4, mammals possess a  $\Delta^9$ -desaturase, and the ability to convert VA to RA has been demonstrated for several species, including humans (Turpeinen *et al.*, 2002; see also review by Palmquist *et al.*, 2005). In addition, Banni *et al.* (2001) observed that feeding rats increasing amounts of pure VA resulted in a progressive increase in tissue concentration of RA, and a corresponding reduction in the number of premalignant mammary lesions, an early marker for mammary tumors. Subsequent investigations established that dietary VA derived from VA/RA-enriched butter also resulted in a dose-dependent increase in the accumulation of CLA in the mammary fat pad, which was accompanied by a parallel decrease in tumor incidence and tumor number (Corl *et al.*, 2003), and that the anticarcinogenic effects of VA were predominately, perhaps exclusively, mediated through its conversion to RA *via*  $\Delta^9$ -desaturase (Lock *et al.*, 2004). Therefore, VA and RA derived from milk fat are both anticarcinogenic and this series of pre-clinical investigations clearly demonstrate the feasibility of a functional food approach using dairy products enriched in VA and RA in the prevention of mammary cancer.

Premalignant lesions and tumors grow when the rate of cell proliferation exceeds cell death, and investigations to date suggest that the

**Table 3.3.** Bioassay of mammary cancer prevention in rats fed different sources of conjugated linoleic acids\*. Adapted from Ip *et al.* (1999)

Dietary treatment	Total CLA in diet (%)	CLA content ( $\mu\text{g}/\text{mg}$ lipid)		Mammary tumors	
		Plasma	Mammary fat	Incidence	Total No.
Control butter	0.1	5.4 <sup>a</sup>	7.2 <sup>a</sup>	28/30 <sup>a</sup> (93%)	92 <sup>a</sup>
High CLA butter	0.8	23.3 <sup>c</sup>	36.5 <sup>c</sup>	15/30 <sup>b</sup> (50%)	43 <sup>b</sup>
Control butter & synthetic CLA	0.8	18.4 <sup>c</sup>	26.2 <sup>b</sup>	16/30 <sup>b</sup> (53%)	46 <sup>b</sup>

\* Dietary treatments were initiated at weaning and continued for 30 days. All animals were then injected with methylnitrosourea (MNU) to induce mammary tumors and switched to a 5% corn oil diet with no CLA. They remained on this diet for 24 weeks and were then sacrificed for tissue analysis. Values with unlike superscripts in the same column (a, b, c) differ ( $P < 0.05$ ).

anticarcinogenic effects of CLA involve a multitude of mechanisms. These include a decrease in cell proliferation, an increased rate of apoptosis, inhibition of angiogenesis, modulation of the immune cell environment, alteration in the eicosanoid signalling pathways and a possible antioxidant role (see Belury, 2002; Banni *et al.*, 2003; Ip *et al.*, 2003). Particular mechanisms may vary in importance depending on the tissue-specific process being regulated, and the opportunity to exploit the diversity in the mechanism of action of CLA may form the basis for the range in tissues and cancer types in which CLA is effective.

Evaluating the specific role of CLA in health maintenance and the prevention of cancer in humans is difficult. Since cancer takes many years to develop, documenting that dietary CLA is beneficial in health maintenance and the prevention of this disease is a major challenge. Results, from epidemiological studies gave conflicting results (Aro *et al.*, 2000; Voorrips *et al.*, 2002; Chajes *et al.*, 2003; McCann *et al.*, 2004). This inconsistency is not surprising. Dairy products are used in recipes for many manufactured food products, and estimating CLA intake is further complicated by the fact that CLA is a fatty acid and dairy products vary widely in fat content, milk fat varies widely in CLA content, and analysis of RA is difficult and reported values are often inaccurate (see reviews by Parodi, 2004; Bauman *et al.*, 2005). Another approach would be dietary interventions using biomarkers as end points to predict reduced cancer risk, but to date there are no consensus biomarkers for breast cancer and many other cancer types. Clearly, assessing the role of dietary CLA in functional foods for the prevention of cancer presents some unusual difficulties, and thus many of the traditional approaches to evaluate human health effects have substantial limitations.

### 3.6.2.2. Atherosclerosis

Investigations of the effects of CLA on atherosclerosis are limited compared with anticarcinogenic studies. A number of animal studies have demonstrated that dietary supplementation with mixtures of CLA isomers can reduce the development of atherosclerotic lesions (Lee *et al.*, 1994; Nicolosi *et al.*, 1997; Kritchevsky *et al.*, 2000; 2002; Wilson *et al.*, 2000) and even induce the regression of pre-existing lesions in rabbits (Kritchevsky *et al.*, 2000; 2004). However, one study, in the atherosclerosis-susceptible C57BL/6 mouse, showed that CLA had no effect on atherosclerotic lesions, and could even promote their development (Munday *et al.*, 1999). Studies with pure isomers recently demonstrated that RA and *trans*-10, *cis*-12 CLA were equally effective in reducing cholesterol-induced atherogenesis in rabbits (Kritchevsky *et al.*, 2004). Of particular significance is that RA induced the regression of atherosclerotic lesions in the ApoE<sup>-/-</sup> knockout mouse (Toomey *et al.*, 2003). This model has been used widely in studies of atherosclerosis because it spontaneously develops lesions on a regular low-fat, low-cholesterol diet with a histopathology similar to lesions that develop in humans (Meir and Leitersdorf, 2004).

Changes in both total plasma cholesterol and individual lipoprotein cholesterol concentrations have been implicated as major determinants of the risk of atherosclerosis and this has led to a number of studies which specifically investigated the effects of CLA on cholesterol and lipoprotein metabolism in animal models. Most have used a synthetic source composed of a mixture of CLA isomers, and results have been inconsistent with some showing beneficial changes in blood lipid variables while others have shown no effect (see Bauman *et al.*, 2005). We recently completed a study using the Golden Syrian hamster to examine the potential of CLA when fed as a component of a functional food (VA/RA-enriched butter) as part of a diet that was high in cholesterol (0.2%) and fat (20%). Compared with the control animals, those fed the VA/RA-enriched butter showed a number of beneficial effects, including reduced total plasma cholesterol and VLDL and LDL cholesterol lipoproteins, suggesting that CLA may modify the production of atherogenic lipoproteins by the liver (Lock *et al.*, 2005b). In addition, the VA/RA-enriched butter produced a less atherogenic profile than an equivalent diet in which the VA/RA-enriched butter was replaced by *trans* fatty acids from partially hydrogenated vegetable oil (Lock *et al.*, 2005b). Consistent with these findings, it has been proposed (McLeod *et al.*, 2004) that RA could, in the absence of other CLA isomers, improve hepatic lipid metabolism. This may explain why VA/CLA-enriched butter elicited such impressive effects compared to studies in which synthetic CLA isomer mixtures are used, since naturally-derived sources of CLA provide essentially only RA.

It is important to note that an elevated and/or altered plasma lipid level is only one of a wide range of risk factors that contribute to the clinical manifestations of cardiovascular disease in humans (Lusis, 2000). Consequently, in some studies, the reduced incidence of atherosclerosis in animals fed CLA was not accompanied by an improvement in the plasma lipid profile during the CLA feeding phase (Wilson *et al.*, 2000). Reasons for these effects are not understood fully. However, atherosclerosis can also be considered as a chronic inflammatory disease (Libby, 2002) and several important anti-inflammatory effects have been associated with the use of RA; these include a reduction in the expression of COX-2, PGE<sub>2</sub>, reduced release of nitric oxide, a decreased production of pro-inflammatory cytokines, and PPAR $\gamma$  activation (Urquhart *et al.*, 2002; Yu *et al.*, 2002; Toomey *et al.*, 2003).

Since results from studies with biomedical models indicate potential, there is of obvious interest in the effects of RA consumption in foods on the risk of atherogenesis in humans. The use of surrogate biomarkers for disease risk is more readily achievable for atherosclerosis than for cancer in humans and a number of genetic and environmental risk factors have been identified, with the relative abundance of the different lipoproteins being of primary importance (Lusis, 2000). To date, there have been no epidemiological studies that have examined the intake of CLA derived from foods with the risk of atherosclerosis. However, as discussed in Section 3.6.2.1, the challenge of adequately evaluating the effect of dietary intake of CLA from different food sources presents some special limitations.

Several human intervention studies involving dietary supplements of CLA in the form of capsules have shown plasma lipid variables as secondary observations, but most utilized mixed isomers of CLA and gave variable results (see Bauman *et al.*, 2005). However, two recent studies examined the specific effects of RA on blood lipids in healthy subjects; a CLA supplement containing a 50:50 mixture of RA and *trans*-10, *cis*-12 CLA significantly improved plasma triacylglycerol and VLDL metabolism, with an 80:20 CLA isomer blend (RA:*trans*-10, *cis*-12 CLA) significantly reducing the concentration of VLDL-cholesterol, providing further evidence for the role of RA in altering hepatic lipid metabolism (Noone *et al.*, 2002). Utilizing pure CLA isomers, it was observed that RA and *trans*-10, *cis*-12 CLA had opposing effects on blood lipids in healthy humans; plasma triacylglycerol, total plasma cholesterol, LDL-cholesterol and LDL:HDL-cholesterol were all lower during supplementation with RA compared to *trans*-10, *cis*-12 CLA (Tricon *et al.*, 2004). A companion study showed that CLA isomers were readily incorporated into plasma and cellular lipids to a similar extent and in a dose-dependent manner (Burdge *et al.*, 2004). Although these data are limited, they provide support that some of the anti-atherosclerosis effects

of CLA reported in animal models will extend to humans. As previously mentioned, often in atherosclerosis studies utilizing animal models, the beneficial effects did not involve alterations in plasma lipids. Thus, CLA studies in humans that focus on the possible role of eicosanoid- and cytokine-related effects could also be of importance.

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# Intracellular Origin of Milk Fat Globules and the Nature of the Milk Fat Globule Membrane

T.W. Keenan and I.H. Mather

## 4.1. Introduction

In this chapter, we review what is known about the intracellular origin, growth, cytoplasmic transit and secretion of the lipid globules of milk, and the nature and intracellular origin of the milk fat globule membrane (MFGM). Material found in the MFGM appears to originate from the endoplasmic reticulum during the initial formation of the lipid droplet precursors of milk fat globules, and from post-Golgi membranes, including the apical plasma membrane, during the secretion of lipid droplets from the cell. Milk fat globules constitute 95%, by weight, of the lipids in cow's milk, of which more than 98% comprise triacylglycerols. The remainder of the mass of fat globules is composed of diacylglycerols, sterols, sterol esters, phospholipids, glycosphingolipids, and proteins associated with the droplet surface.

In writing this chapter we have emphasized findings since the previous edition of this book was published in 1994. Much of the new information gained in this area concerns the proteins of the MFGM. Since the previous edition, there have been few additional studies on intracellular aspects of milk lipid globule formation and on the composition of the MFGM. For those interested in earlier literature and coverage of historical aspects of research on milk lipid globules, the comprehensive review by Brunner (1974) and later reviews (Anderson and Cawston, 1975; Patton and Keenan, 1975;

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

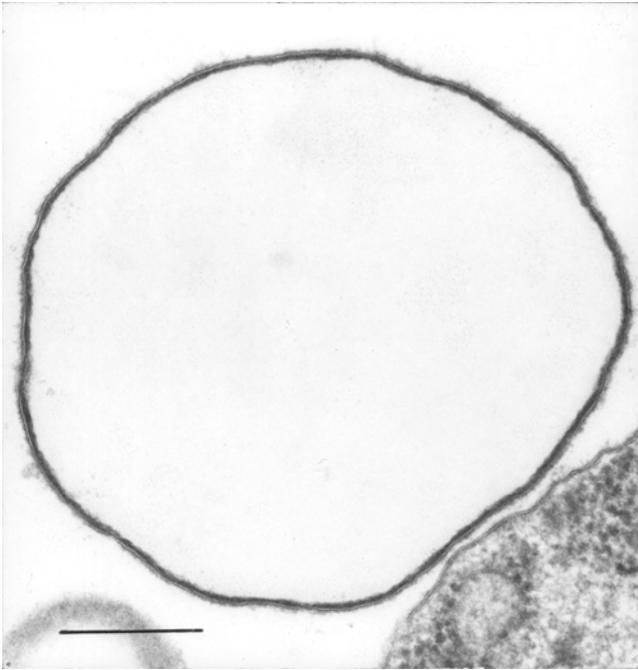
McPherson and Kitchen, 1983; Keenan *et al.*, 1988; Mather, 1987; Keenan and Dylewski, 1995; Keenan and Patton, 1995; Mather and Keenan, 1983, 1998) are recommended.

Much of the information we have presented on MFGM has been learned through studies of cows' milk. Milk fat globules and MFGM from other species, notably from humans, have been subjects of increasing study over the past 20 years. Herein, we discuss information gained from other species only where equivalent information for the cow is not available, or where information from other studies is contradictory to, or confirmatory of, what is believed about cows' milk. Extensive information about human milk fat globules can be found in books edited by Jensen (1995) and Newburg (2001).

## 4.2. Intracellular Origin and Growth of Milk Fat Globules

The membrane surrounding milk lipid globules essentially is a tripartite structure that originates from the apical plasma membrane, from the endoplasmic reticulum and possibly from other intracellular compartments. That portion of the MFGM originating from apical membranes, termed the primary membrane, has a typical bilayer appearance and electron-dense coat material on the inner face (Figure 4.1). The material derived from the endoplasmic reticulum has the appearance of a monolayer of proteins and polar lipids that covers the triacylglycerol-rich core lipids of the globule before secretion (Figure 4.2). This coat material compartmentalizes the core lipids within the cell and may participate in the intracellular fusion of droplets with each other. Constituents of this coat also may be involved in the interaction of droplets with the plasma membrane during the process of secretion.

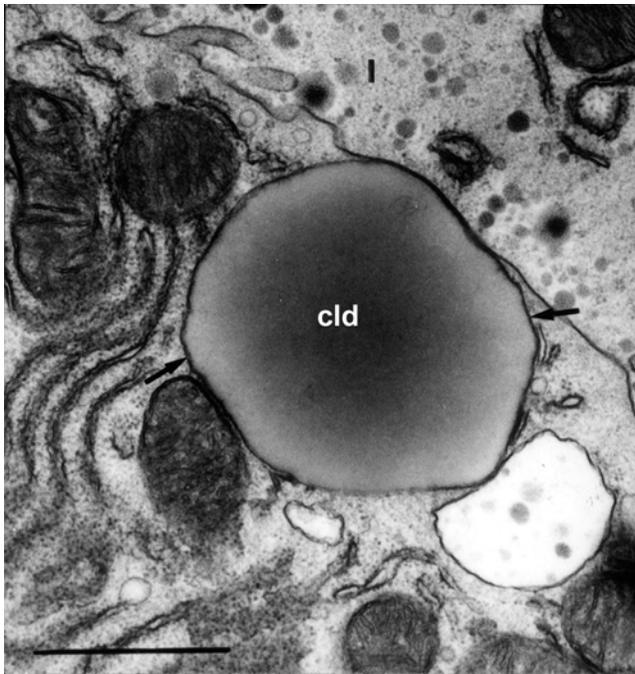
Small lipid droplets that are the intracellular precursors of milk lipid globules appear to accumulate at focal points on or in the endoplasmic reticulum membrane (Dylewski *et al.*, 1984a; Zaczek and Keenan, 1990). Several studies suggest that the lipids, presumed to be primarily triacylglycerols, accumulate between the outer and inner halves of the bilayer and are then released into the cytosol as droplets coated with the cytoplasmic half of the endoplasmic reticulum membrane (Patton and Keenan, 1975; Zaczek and Keenan, 1990; Keenan *et al.*, 1992; Mather and Keenan, 1998). Storage lipid droplets in adipocytes and other cells also appear to be released from the endoplasmic reticulum into the cytosol in like manner (Murphy and Vance, 1999; Murphy, 2001). Using a cell-free system, Keenan *et al.* (1992) showed that lipid droplets are released from mammary gland endoplasmic reticulum bound to nitrocellulose membrane and that these droplets in



**Figure 4.1.** Electron micrograph of a milk fat globule showing the nature of the surrounding membrane. The membrane has a typical bilayer appearance and densely staining material coating the inner face. Bar = 0.1  $\mu\text{m}$ . Reproduced from Keenan *et al.* (1988) with permission. Micrograph courtesy of W.W. Franke.

morphology and composition resemble droplets formed *in situ*. Using a proteomics approach to identify proteins associated with cytosolic lipid droplets, Wu *et al.* (2000) obtained evidence consistent with the interpretation that lipid droplets in milk originate from the endoplasmic reticulum.

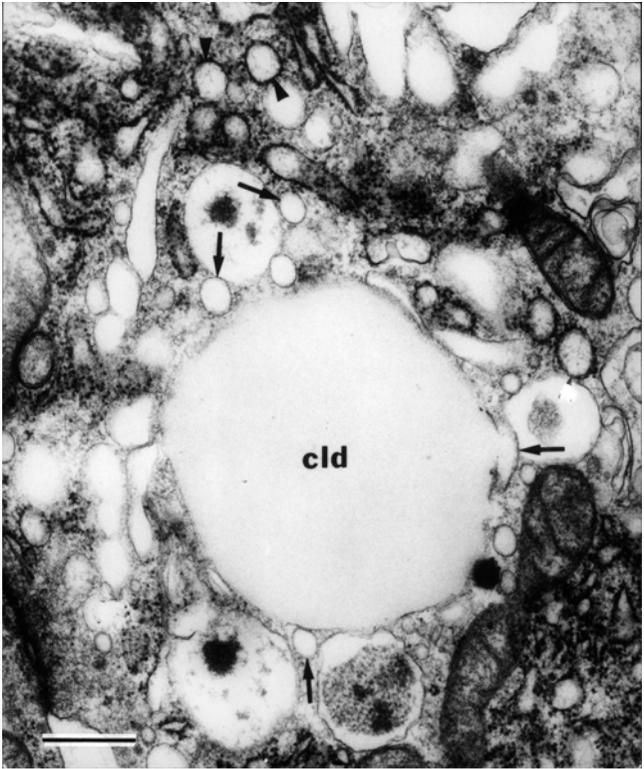
Milk lipid globule precursors are present in the cytosol as droplets ranging in diameter from less than 0.5 to more than 4  $\mu\text{m}$  (Dylewski *et al.*, 1984a; Deeney *et al.*, 1985). These precursors appear to originate as small droplets, with diameters under 0.5  $\mu\text{m}$ , termed microlipid droplets. Droplets grow in volume by fusing with each other, giving rise to larger droplets, termed cytoplasmic lipid droplets (Figure 4.3). In a cell-free system, droplet fusion was promoted by calcium and by an, as yet, uncharacterized high molecular weight protein fraction from cytosol. Gangliosides appear to be involved in this fusion in some manner (Valivullah *et al.*, 1988). While small droplets fused readily, larger cytoplasmic lipid droplets did not fuse in the



**Figure 4.2.** Electron micrograph showing a cytoplasmic lipid droplet (cld) in a cell fixed with potassium ferrocyanide. Dark-staining material of variable thickness coats the surface of the droplet (arrows). The alveolar lumen (l) is denoted. Bar = 1  $\mu$ m. From Dylewski *et al.* (1984a) with permission.

cell-free system. The reasons for this are not apparent but may be related to some compositional differences between the coat material on micro-plasmic and cytoplasmic lipid droplets (Dylewski *et al.*, 1984a; Deeney *et al.*, 1985). Droplets of all sizes have a triacylglycerol-rich core surrounded by a protein and polar lipid coat. In composition, the protein and polar lipid material surrounding both micro-plasmic and cytoplasmic lipid droplets is similar but not identical.

While available evidence supports the view that an increase in the volume of lipid droplets occurs through fusion of lipid droplets, it is by no means certain that this is the only mechanism supporting droplet growth. Other possibilities include, e.g., growth *via* lipid transfer proteins that convey triacylglycerols from their site of synthesis to lipid droplets (Patton, 1973). Some 4% or more of the total lipid in lactating rat mammary gland is found in the cytosol and much of this complement of cytosolic lipid is



**Figure 4.3.** Electron micrograph showing a cytoplasmic lipid droplet (cld) and numerous microlipid droplets, some of which are denoted by arrows. An apparent fusion figure between the cld and a microlipid droplet is denoted by the arrow on the right. The specimen was fixed simultaneously with glutaraldehyde and osmium tetroxide. Bar = 0.5  $\mu\text{m}$ .

associated with the fatty acid synthase complex (Keon *et al.*, 1993). In a cell-free system, fatty acid synthase was able to transfer lipids to microlipid droplets and to the endoplasmic reticulum (Keon *et al.*, 1993, 1994). Whether this transfer occurs in cells and supports droplet formation or growth remains to be determined. Further, whether droplet growth is purely random or is a regulated process is unknown. Monomeric GTP-binding proteins known to be involved in vesicular interactions, *arf*, *rab 3a*, and *rab 1a* or *b*, are associated with lipid droplets (Ghosal *et al.*, 1993; T. W. Keenan, unpublished). This suggests the possibility that fusion may be regulated but this remains to be tested experimentally.

### 4.3. Intracellular Transit of Lipid Droplets

Lipid droplets migrate from their sites of origin, mostly in basal cell regions, to the apical pole of the cell, from whence they are secreted as milk lipid globules. Details of the guiding mechanism of this migration remain speculative but it is probable that cytoskeletal elements are involved. Droplets appear to transit exclusively toward the apical cytoplasm and bypass the supranuclear secretory cone formed by interconnected dictyosomes of the Golgi apparatus (Dylewski *et al.*, 1984b). However, there is no morphological evidence for specific contacts between lipid droplets and elements of the cytoskeleton.

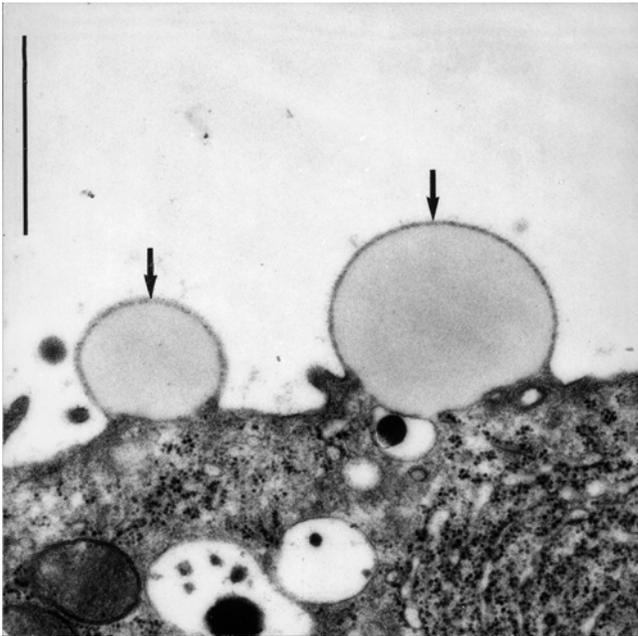
Microtubules are abundant in milk-secreting cells (Nickerson and Keenan, 1979) and, in guinea pigs, the tubulin content of mammary epithelial cells increases substantially during late pregnancy and the first half of lactation (Guerin and Loizzi, 1980). Attempts to interfere with microtubule assembly using colchicine and vinblastine have yielded contradictory results. Intramammary infusion of colchicine into lactating goats reversibly suppressed milk secretion (Patton, 1974). Colchicine did not appear to alter the rate of lipid synthesis in goats and, in treated glands, lipid droplets were larger than those in the contralateral untreated gland (Patton *et al.*, 1977). However, in the foregoing and in other studies, secretion of the serum phase of milk was suppressed also (Henderson and Peaker, 1980; Nickerson *et al.*, 1980). Addition of colchicine to tissue slices from lactating sheep and rabbits inhibited protein secretion but had no apparent effect on lipid secretion (Daudet *et al.*, 1981). In one study, intramammary infusion of colchicine into goats was found to suppress mammary extraction of constituents from serum and to suppress the rate of milk synthesis as well (Henderson and Peaker, 1980). Based on these, in part, disparate observations, and on the potential non-specific effects of colchicine and vinblastine (discussed in Mather and Keenan, 1983, 1998), we cannot ascribe a specific role to microtubules in milk fat secretion.

Actin-containing microfilaments are abundant in milk-secreting cells and are concentrated in apical cell regions (Amato and Loizzi, 1981). No actin staining was observed on luminal aspects in regions where fat droplets were in close apposition to the apical plasma membrane (Franke *et al.*, 1981). However, actin has been localized by immunofluorescence microscopy in the cytoplasmic surface along basal regions of budding lipid droplets. This observation raises the possibility that filamentous actin may form part of a contractile apparatus that functions in lipid secretion (Keenan *et al.*, 1988), a possibility that has yet to be investigated. Recently, and in contrast to a

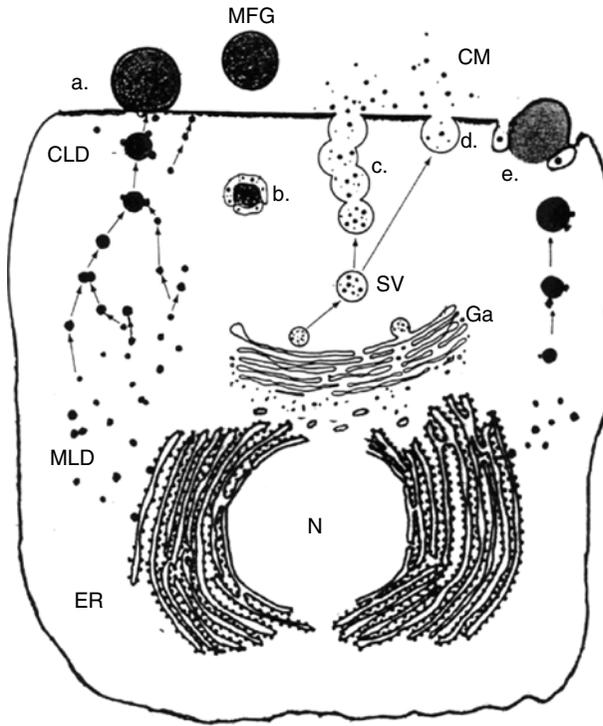
previous finding (Keenan *et al.*, 1977), actin has been identified, immunologically, as a constituent of the MFGM (Heid and Keenan, 2005).

#### 4.4. Secretion of Milk Fat Globules

Lipid droplets are secreted enveloped by cellular membranes. As first recognized by Bargmann and Knoop (1959), lipid droplets approach closely to, or contact, the apical surface and are gradually enveloped in membrane up to the point at which they dissociate from the cell, surrounded by plasma membrane (Figure 4.4; mechanism a, Figure 4.5). This is the widely-accepted mechanism of milk fat globule secretion (Patton and Keenan, 1975; Mather and Keenan, 1983, 1998; Keenan *et al.*, 1988; Keenan and Patton, 1995). Wooding (1971a, 1973) and others (Kralj and Pipan, 1992) obtained morphological evidence for an alternative mechanism based on an observed association between lipid droplets and secretory vesicles in apical cell regions



**Figure 4.4.** Electron micrograph showing two small lipid droplets (arrows) which, apparently, were in the process of being secreted. These droplets are partly enveloped in what appears to be plasma membrane in the apical cell region. Bar = 1  $\mu\text{m}$ . From Deeney *et al.* (1985) with permission.



**Figure 4.5.** Diagrammatic summary of what is known about the intracellular origin, growth, and secretion of milk fat globules. (a) Secretion of a milk fat globule by envelopment in apical plasma membrane. (b) As originally proposed by Wooding (1971a), under certain conditions secretory vesicles may surround fat droplets and fuse together to form a vacuole containing the fat droplet. Presumably, these vacuoles are transported to the apical surface where the vacuolar contents are released by exocytosis. (c) Secretion of the serum (non-fat) phase of milk by compound exocytosis. (d) Secretion of the serum phase of milk by simple exocytosis. (e) A possible but as yet undocumented combination of apical plasma membrane and secretory vesicle membrane mechanisms for secretion of fat globules. Abbreviations: CLD, cytoplasmic lipid droplet; CM, casein micelle; ER, endoplasmic reticulum; Ga, Golgi apparatus; MLD, microlipid droplet; MFG, milk fat globule; N, nucleus; SV, secretory vesicle.

(see also Franke and Keenan, 1979; Stemberger *et al.*, 1984). Wooding (1971a, 1973) proposed that progressive fusion of neighboring vesicles on the surfaces of lipid droplets may lead to the formation of intra-cytoplasmic vacuoles containing both casein micelles and lipid droplets enveloped with secretory vesicle membrane (mechanism b, Figure 4.5). The content of such vacuoles may then be released from the cell by exocytosis. Wooding (1973) suggested that vacuole formation may occur *in vivo* at certain stages of the

secretory cycle, although these observations were made with specimens that may have been fixed for microscopic examination under less than optimal conditions. Kralj and Pipan (1992) provided evidence suggesting that vacuolar lipid secretion may be common both during the parturient period and when milk secretion is inhibited temporarily.

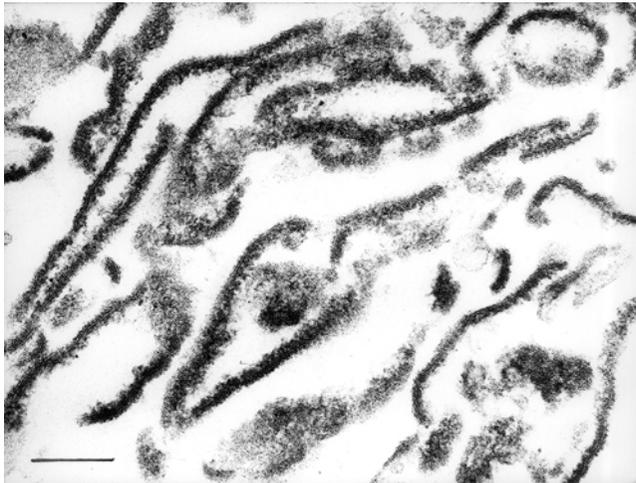
By virtue of their static nature, electron micrographs provide no information on the kinetics of secretion. Just the process of fixation may provoke a temporary decrease in secretory events and a consequent accumulation of lipid droplets and secretory vesicles in cells before they succumb to the fixative agent. Such crowding in the apical cytoplasm could well lead to promiscuous associations and interactions that are unrelated to normal secretory processes (Mather and Keenan, 1998).

Contributions to the MFGM from the apical plasma membrane and secretory vesicle membrane could be assessed by direct biochemical comparisons of these membranes isolated from mammary homogenates. However, technical challenges inherent to the isolation of secretory vesicle and apical plasma membrane have yet to be overcome. Well-characterized apical plasma membrane preparations from mammary gland have not been forthcoming; preparations of plasma membranes from this source are enriched in baso-lateral membrane fragments and may contain plasma membranes from other cell types (Keenan *et al.*, 1970, 1989). Nevertheless, comparisons of plasma membranes isolated from mammary gland with MFGM show these membranes to be similar in polar lipid composition and some protein constituents (Keenan *et al.*, 1970, 1989). Secretory vesicles from the mammary gland are osmotically fragile and have proven difficult to isolate. Preparations that have been described differ substantially from the MFGM (Sasaki *et al.*, 1978; Keenan *et al.*, 1979). However, these preparations appear to be enriched in immature vesicle membranes, which may have a different composition from the membranes of mature secretory vesicles. Based on these considerations, no realistic interpretations about the plasma membrane or secretory vesicle membrane origin of MFGM can be made from the extant biochemical data (Mather and Keenan, 1998).

Available cytochemical evidence favors plasma membrane envelopment as the principal mechanism for the secretion of milk fat globules. Butyrophilin (BTN), a major integral membrane protein of milk fat globules, is expressed on the apical plasma membrane (Franke *et al.*, 1981) and appears to be concentrated in areas of the membrane associated with budding lipid droplets (Mather, 1987). By immunoperoxidase labeling, BTN was not detected on intracellular membranes (Franke *et al.*, 1981). The MUC1 mucin also is concentrated in apical regions of the plasma membrane and in MFGM (Johnson and Mather, 1985; Mather *et al.*, 2001). In contrast, secretory vesicle membranes contain only about 10% of the amount of MUC1 estimated, by

immunogold labelling, to be associated with apical plasma membrane and the MFGM. These observations make it unlikely that substantial portions of the MFGM originate from secretory vesicle membranes. Finally, more than 80% of the total number of fat globules in cows' milk are less than  $1\ \mu\text{m}$  in diameter (Mulder and Walstra, 1974), a size incompatible with the direct involvement of mature secretory vesicles in the secretion of these small globules. Morphological evidence suggests that these small globules are secreted by envelopment with plasma membrane (Figure 4.4).

During the budding of lipid droplets from the apical surface, a uniform distance of 10 to 20 nm is maintained between the outer surface of the droplet and the cytoplasmic face of the plasma membrane (Wooding, 1971a). This intervening space appears in micrographs to be filled with an electron-dense material that may originate from the cytoplasm or from the inner face of the apical plasma membrane. During secretion, this material is most likely carried over into milk fat globules and gives rise to the characteristic coat visible as a fuzzy, amorphous layer on the inner face of the MFGM isolated from cream (Wooding and Kemp, 1975; Freudenstein *et al.*, 1979) (Figure 4.6). The origin and nature of this material was the subject of much speculation, primarily because understanding its origin and nature appeared to be a key towards understanding the mechanism for milk fat



**Figure 4.6.** Electron micrograph of MFGMs which were released from washed milk fat globules by churning and collected by ultracentrifugation. These membranes occur mostly as sheets and show little tendency to vesiculate. Membranes retain the dense-staining protein coat material that originally was oriented toward the globule interior. Bar =  $0.2\ \mu\text{m}$ .

globule secretion. Studies using various extractants established that the MFGM coat was primarily proteinaceous in nature (Wooding and Kemp, 1975; Freudenstein *et al.*, 1979) and freeze-etch micrographs have shown that it exists in a paracrystalline hexagonal array with long-range order (Wooding, 1977; Buchheim, 1982).

Recent work on the characterization of proteins of the MFGM has done much to reveal the nature of this coat material. From a consideration of protein topologies and protein-protein interactions, it is likely that the protein coat comprises the cytoplasmic tail of BTN, a type 1 transmembrane glycoprotein, the peripheral protein xanthine dehydrogenase/oxidoreductase (XDH), and the intracellular lipid-droplet-associated protein adipophilin (ADPH, known also as adipocyte differentiation-related protein or ADRP) (for reviews, see Mather and Keenan, 1998; Mather, 2000). There is substantial evidence that BTN and XDH interact directly with each other. In cows, these two proteins are expressed in constant molar proportions throughout lactation (Mondy and Keenan 1993) and they can be covalently bound to each other by bifunctional cross-linking reagents (Valivullah and Keenan, 1989). The cytoplasmic tail of BTN, expressed as a recombinant fusion protein in *E. coli*, specifically binds to XDH *in vitro* (Ishii *et al.*, 1995). XDH can be released from the MFGM by the reduction of disulfide bonds (Spitzberg and Gorewit, 1998) and proteins in the detergent-insoluble membrane coat complex can be dissociated by exogenous thiol reagents (I.H. Mather and T.W. Keenan, unpublished). This suggests that interactions between XDH and BTN are promoted, or stabilized, by disulfide bonds.

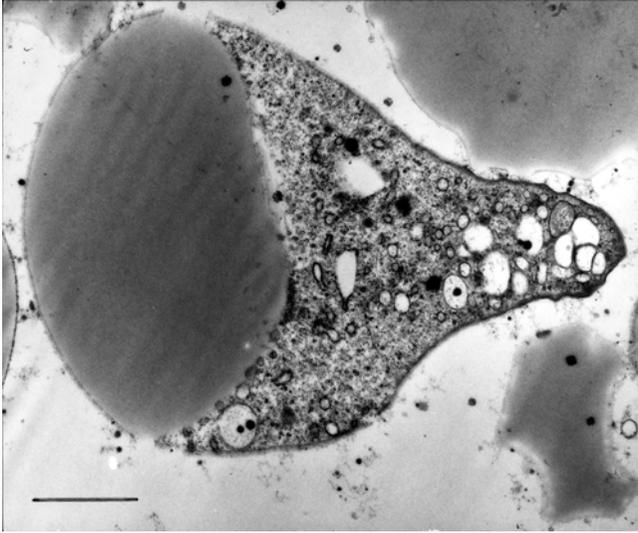
Direct evidence that XDH and BTN are essential for normal milk lipid secretion has recently been provided by the use of gene-targeting techniques (Vorbach *et al.*, 2002; Ogg *et al.*, 2004). Heterozygous mice with one ablated XDH allele (*Xdh*<sup>+/-</sup>) displayed deficiencies in lipid secretion with consequent accumulation of lipid within mammary epithelial cells. The milk of these animals is characterized by the presence of large lipid droplets with disrupted membranes. Lactation in mice with both XDH alleles ablated (*Xdh*<sup>-/-</sup>) could not be studied because such mice did not survive beyond six weeks of age (Vorbach *et al.*, 2002). The same phenotype was observed in mice in which BTN expression was either disrupted or eliminated (Ogg *et al.*, 2004). We propose that interactions between a BTN/XDH complex and other proteins on the intracellular lipid droplet surface mediate interactions between lipid droplets and membrane (Mather and Keenan, 1998). Since XDH is a homodimer, binding to BTN may initially induce the formation of BTN dimers which could further aggregate through adhesive interactions to form large complexes of the type observed in freeze-etch micrographs. Alternatively, BTN may first self-aggregate in the plane of the membrane

bilayer and bind to XDH homodimers (A. Rao and I.H. Mather, unpublished observations). Such protein-protein interactions could well explain the progressive envelopment of lipid droplets by plasma membrane, although this remains speculative. Disulfide bond formation also may play a role, since protein disulfide isomerase has been identified as a constituent protein of intracellular lipid droplets (Ghosal *et al.*, 1994; Wu *et al.*, 2000) and, as discussed above, XDH can be released from the MFGM by thiol reagents.

That other proteins are associated with the MFGM coat is probable, particularly proteins associated with the surface of intracellular lipid droplets. However, several of the proteins identified as being associated with intracellular lipid droplets (Wu *et al.*, 2000) have yet to be identified as constituents of the MFGM coat. Two proteins associated with intracellular lipid droplets, protein disulfide isomerase (Ghosal *et al.*, 1994) and the nuclear coactivator protein p100 (Keenan *et al.*, 2000) are absent from MFGM preparations. Thus, there apparently is some selectivity in which of the proteins associated with intracellular lipid droplets are secreted.

The mechanisms controlling milk fat secretion remain unknown. Protein kinases may be implicated because the release of lipid from primary cultures of rat mammary epithelial cells was stimulated by protein kinases (Rohlfis *et al.*, 1993; Spitsberg and Gorewit, 1997) or phosphatases (review, Keenan *et al.*, 1988) are associated with MFGM preparations. CD36, a component of the MFGM, may associate with src family protein kinases in a number of cell types and play some role in intracellular signalling (Greenwalt *et al.*, 1992). That monomeric GTP-binding proteins of the type known to be involved in vesicle-membrane interactions are associated with intracellular lipid droplets and the MFGM suggests a potential regulatory function for G-proteins in milk fat secretion, particularly since a non-hydrolyzable analogue of GTP was shown to stimulate fat secretion from permeabilized rat mammary acini (Ghosal *et al.*, 1993). However, due to lack of any specific information, the control or regulation of milk fat secretion remains an area purely of speculation. What currently is known about the origin, intracellular growth, and secretion of milk fat globules is summarized in Figure 4.5.

During the secretion process, milk fat globules usually are enveloped compactly by membrane but in some instances closure of the membrane entrains some cytoplasm between the membrane and the fat droplet surface. The result is an extracellular fat globule with a so-called signet or crescent of cytoplasm attached (Figure 4.7). These crescents can vary from thin slivers of cellular material to large inclusions that exceed the volume of the fat globule core. Wooding (1977), in his alternative scheme for fat globule secretion, suggested that crescents originate from the trapping of cytoplasm as secretory vesicles fuse around the droplets during vacuole formation.



**Figure 4.7.** Electron micrograph of a milk fat globule in the alveolar lumen that has a large cytoplasmic crescent entrained between the globule and the surrounding membrane. This crescent contains secretory vesicles, ribosome-studded vesicles of apparent endoplasmic reticulum origin, and an abundant amount of particulate material. Bar = 2  $\mu$ m.

Huston and Patton (1990) suggested that an abnormality in the protein coat along the cytoplasmic face of the apical plasma membrane may be responsible for the phenomenon of crescent formation. Inadequate amounts of proteins forming this coat or abnormalities in the distribution of the coat complex may interfere with adhesion of the membrane to the droplet.

Crescents have been observed, morphologically, to contain nearly all intracellular membranes and organelles of the milk-secreting cell, except nuclei. The proportion of globules with crescents appears to vary both between species and in individuals within a species. Between 1 to 5% of the globules in goat and guinea pig milks and about 1% of the globules in cows' milk were observed to contain crescents (Wooding *et al.*, 1970; Huston and Patton, 1990). Janssen and Walstra (1982) measured the quantity of crescent material in the milk of cows, goats, rats, pigs, sheep, rabbits, and humans and found that cows have the lowest, and rabbits have the highest, quantity of fat globule-associated crescent material. In humans, Patton and Huston (1988) found a diurnal variation in crescent frequency.

Gaining an understanding of the molecular mechanism of crescent formation would advance our understanding of the process of milk fat globule secretion. Crescents are a route by which cellular constituents can

enter milk. This may be a route for the entry of bioactive molecules such as hormones or growth factors into milk.

#### 4.5. Isolation and Gross Composition of MFGM

Some of the membrane that initially surrounds fat globules is lost following secretion, either within the mammary gland or in expressed milk (Wooding, 1971b, 1974). Estimates of the extent of this loss vary considerably (Mather and Keenan, 1983). That changes occur also in MFGM after secretion is probable, but there are few quantitative data available on these changes. Clearly, the manner in which milk is collected and handled also may cause loss and compositional alteration of the MFGM, i.e., the nature of the MFGM as isolated and studied may not necessarily reflect the membrane as it was when fat globules were secreted initially (for review, see Evers, 2004).

Membranes can be released from fat globule suspensions by several methods, including churning (agitation), freezing and thawing, treatment with detergents, or by suspension in polar, aprotic solvents. Once released, MFGM can be collected readily by centrifugation at a high centrifugal force or, after inducing membrane aggregation, at a lower force. Details of these methods and the effects of the preparative method on the composition of the resultant MFGM have been reviewed and will not be repeated here (Keenan *et al.*, 1988; Keenan and Dylewski, 1995).

Information on the gross composition of MFGM is summarized in Table 4.1. Proteins and lipids account for 90%, or more, of the dry weight of the MFGM. However, reported amounts of the total membrane weight

**Table 4.1.** Gross composition of bovine MFGM<sup>a</sup>

Constituent class	Unit	Amount
Protein	weight %	25–60
Total lipids	mg/mg protein	0.5–1.1
Phospholipids	mg/mg protein	0.13–0.34
Neutral lipids	mg/mg protein	0.25–0.88
Glycosphingolipids	µg/mg protein	13 <sup>b</sup>
Hexoses	µg/mg protein	108
Hexosamines	µg/mg protein	66
Sialic acids	µg/mg protein	20
Glycosaminoglycans	µg/mg protein	0.1
RNA	µg/mg protein	20

<sup>a</sup> From data compiled and reviewed in Keenan *et al.* (1988) and Keenan and Dylewski (1995).

<sup>b</sup> Calculated assuming average molecular weights of neutral glycosphingolipids and gangliosides of 850 and 1470 Da, respectively.

accounted for by proteins and specific lipids vary considerably. In particular, there is wide variation in reported values for neutral lipid, generally measured so as to include sterols and steryl esters, unesterified fatty acids, and monoacylglycerols, diacylglycerols and triacylglycerols. Much of this reported variation is undoubtedly due to methodological differences in membrane preparation. Hexoses, hexosamines and sialic acids in the MFGM appear to be bound exclusively covalently to proteins and glycosphingolipids. MFGM, like plasma membrane, is enriched in glycosphingolipids.

Several workers have detected RNA in MFGM preparations. Swope and Brunner (1965) estimated that bovine MFGM contains about 20  $\mu\text{g}$  of RNA per mg protein. Jarasch *et al.* (1977) measured about the same amount of RNA but were able to reduce this amount to about 10  $\mu\text{g}/\text{mg}$  protein by extraction of MFGM with high ionic strength buffers. This RNA may originate from ribosomes associated with the surface of lipid droplets within the cell (Dylewski *et al.*, 1984a), or it may be from fragments of endoplasmic reticulum or ribosomes trapped in cytoplasmic crescents. DNA has not been found in MFGM preparations (Jarasch *et al.*, 1977). Why glycosaminoglycans, normal basement membrane constituents, are found in the MFGM remains to be explained. Hyaluronic acid, chondroitin sulfate and heparin sulfate have been identified as constituents of glycosaminoglycan fractions from MFGM (Lis and Monis, 1978; Shimizu *et al.*, 1981).

#### 4.6. Lipid Composition of the MFGM

Triacylglycerols usually are the most abundant lipid class found in MFGM (Table 4.2) but the preparative method has a major influence on the amount in individual preparations, probably because variable quantities of triacylglycerols remain associated with the membrane during isolation. MFGM-associated triacylglycerols contain higher proportions of palmitate and stearate (i.e., long-chain fatty acids with relatively high melting points) than do triacylglycerols of the core fat. However, MFGM was not enriched in high-melting point triacylglycerols when membranes were isolated from fat globules destabilized at an elevated temperature (Vasic and DeMan, 1966; Bracco *et al.*, 1972). Walstra (1974) suggested that high-melting point triacylglycerols originate from fat crystals that adhere to the membrane during cooling and churning. Most triacylglycerols are assumed to be associated with the cytoplasmic face of the MFGM, i.e., with the region in contact with the globule core; the outer face of milk fat globules appears to contain little neutral lipid (Newman and Harrison, 1973).

Monoacylglycerols and diacylglycerols may be genuine membrane constituents or may be products of lipolysis. Unesterified fatty acid levels

**Table 4.2.** Lipid composition of bovine MFGM<sup>a</sup>

Constituent class	Proportion of total lipids (%)
Triacylglycerols	62
Diacylglycerols	9
Monoacylglycerols	Traces
Sterols	0.2–2
Sterol esters	0.1–0.3
Unesterified fatty acids	0.6–6
Hydrocarbons	1.2
Phospholipids	26–31
<i>Constituent class</i>	Proportion of total phospholipids (%)
Sphingomyelin	22
Phosphatidylcholine	36
Phosphatidylethanolamine	27
Phosphatidylinositol	11
Phosphatidylserine	4
Lysophosphatidylcholine	2

<sup>a</sup> From data compiled in Patton and Keenan (1975), Keenan *et al.* (1988) and Dylewski and Keenan (1995).

in the MFGM vary widely between preparations and, again, they may originate by lipolysis. There is extensive variation in the amount of sterols, principally cholesterol, reported to occur in the MFGM. Given the probable plasma membrane origin of the primary MFGM and the fact that plasma membranes are enriched in cholesterol (van Meer, 1989), it is not surprising that the MFGM contains abundant cholesterol. Cholesterol accounts for over 90% of the total sterols in cows' milk (Blanc, 1981); other sterols have been identified but it is not clear whether they are components of the MFGM.

Of the total phospholipid pool in milk, about 60% occurs in the MFGM; the remainder is found primarily associated with the membrane fraction in skim milk (Huang and Kuksis, 1967; Patton and Keenan, 1971). Phospholipids of the fat globule are recovered nearly quantitatively with the MFGM when globules are destabilized at 40°C. The same five major phospholipids present in bovine MFGM (Table 4.2), are found also in the milk or MFGM from several other species with a similar pattern of distribution (for review, see Keenan and Dylewski, 1995). The phospholipid distribution pattern of the MFGM is similar to that of plasma membranes from mammary gland in that the sphingomyelin to phosphatidylcholine ratio is higher than that of intracellular membranes (Keenan *et al.*, 1970, 1988; Kanno, 1990). Lyso-derivatives of phosphatidylcholine and phosphatidylethanolamine are found in the MFGM but in minor amounts in samples handled so as to minimize lipolysis. The proportions of the various phosphoglyceride

classes present in the MFGM as alkyl or alkenyl ethers has not been determined.

The distribution and fatty acid composition of phospholipids in skim milk are similar to those in the MFGM. This has led to the suggestion that these membrane-associated constituents originate from a common cellular source and that the skim milk membranes, which contain much of the skim milk phospholipid pool, may be shed MFGM (for review, see Keenan and Dylewski, 1995). This assumption remains to be tested critically.

Glycosphingolipids are relatively minor constituents of bovine MFGM (Table 4.1) but they have been studied widely because of the known roles of glycosphingolipids and some of their breakdown products in a number of biological phenomena such as growth regulation through modulation of protein kinases and phosphatases (Merrill, 2002; Smith and Merrill, 2002). Bovine MFGM contains two neutral glycosphingolipids, glycosyl-ceramides and lactosyl-ceramides, in nearly equimolar proportions. Neutral glycosphingolipids with more complex carbohydrate chains have not been detected. Nine gangliosides (glycosphingolipids containing at least one molecule of sialic acid) in bovine MFGM have been characterized structurally. The two major gangliosides in bovine MFGM are GD3 and GM3 (nomenclature of Svennerholm, 1963); the other seven gangliosides, in total, account for about 20% of the ganglioside content of the MFGM (for reviews, see Jensen and Newburg, 1995; Keenan and Patton, 1995).

#### 4.7. Enzymes Associated with the MFGM

To date, about 28 different enzymes or enzymatic activities have been detected in MFGM preparations from cows' milk (Table 4.3). Since the compilation in the previous edition of this book (Keenan and Dylewski, 1995), protein kinase activity that phosphorylates some MFGM proteins has been identified in MFGM preparations (Spitsberg and Gorewit, 1997). The NADH oxidase of MFGM, originally identified by its ability to reduce cytochrome c and ferricyanide (Jarasch *et al.*, 1977; Bruder *et al.*, 1978, 1982), was recently shown to have an activity that oscillates with a period of 24 min (Morré *et al.*, 2002).

Some of the enzymes of the MFGM, such as 5'-nucleotidase, adenosine triphosphatase and phosphodiesterase I, are known to be enriched in plasma membranes. However, other enzymes found in the MFGM are known constituents of intracellular membranes or are cytosolic. Why some of these are present in the MFGM remains to be explained; some may possibly originate from material entrained in cytoplasmic crescents and therefore are not true MFGM constituents. Perhaps some enzymes become

**Table 4.3.** Enzymatic activities found in bovine MFGM<sup>a</sup>

Enzymatic activity <sup>b</sup>	EC number
Xanthine oxidoreductase	1.2.3.2
Lipoamide dehydrogenase	1.6.4.3
NADPH oxidase	1.6.99.1
NADH oxidase	1.6.99.3
Sulfhydryl oxidase	1.8.3.2
Catalase	1.11.1.6
$\gamma$ -Glutamyl transpeptidase	2.3.2.1
Galactosyl transferase	2.4.1._
Protein kinase(s) <sup>c</sup>	2.7.1._
Cholinesterase	3.1.1.8
Alkaline phosphatase	3.1.3.1
Acid phosphatase	3.1.3.2
Phosphatidic acid phosphatase	3.1.3.4
5'-Nucleotidase	3.1.3.5
Glucose-6-phosphatase	3.1.3.9
Phosphodiesterase I	3.1.4.1
UDP-glycosyl hydrolases	3.2.1
$\beta$ -Glucosidase	3.2.1.21
$\beta$ -Galactosidase	3.2.1.23
Hexosaminidase <sup>d</sup>	3.2.1.52
Plasmin	3.4.21.7
Inorganic pyrophosphatase	3.6.1.1
Adenosine triphosphatase	3.6.1.3
Thiamine pyrophosphatase <sup>e</sup>	3.6.1.6
Nucleotide pyrophosphatase	3.6.1.9
Aldolase	4.1.2.13
Acetyl-CoA carboxylase <sup>f</sup>	6.4.1.2

<sup>a</sup> Primary references are compiled in Keenan *et al.* (1988), Keenan and Dylewski, (1995) and Mather (2000).

<sup>b</sup> Enzymes are listed by their common names.

<sup>c</sup> Spitsberg and Gorewit (1997).

<sup>d</sup> Kitchen *et al.* (1978).

<sup>e</sup> Sasaki *et al.* (1978).

<sup>f</sup> Enzymatically inactive (Shriver *et al.*, 1989).

adsorbed on the surface of lipid droplets intracellularly and are thus fortuitously associated with secreted fat globules. Several of the enzymes associated with the MFGM have been purified and at least partially characterized; this information has been reviewed by Keenan *et al.* (1988).

Studies on the functional significance of MFGM-associated enzymes have been restricted largely to their involvement in degradative events that affect processing properties or flavor, or for their use as markers of adequate pasteurization. Obviously, enzymes that can produce product defects are of great concern to the dairy industry. Unfortunately, the biological roles

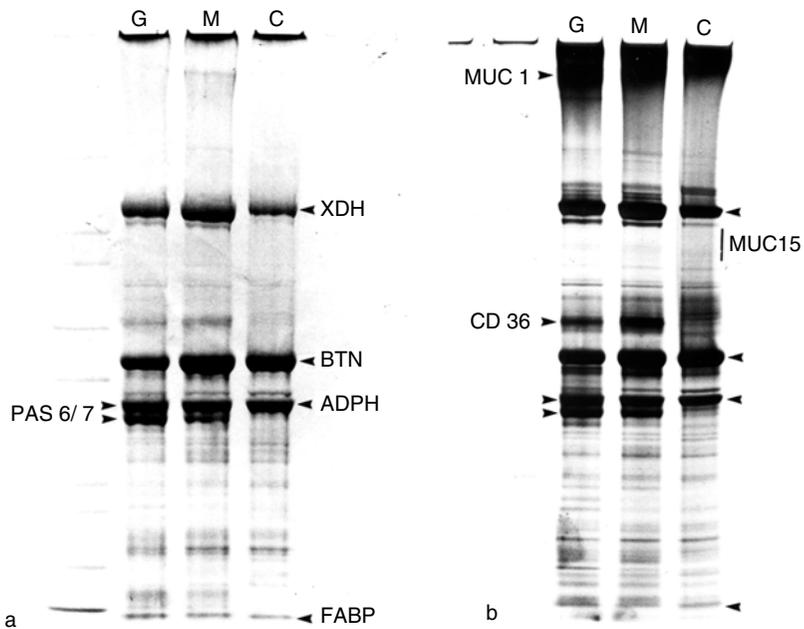
in fat globule formation or secretion, if any, of MFGM-associated enzymes are unknown.

#### 4.8. Proteins of the MFGM

This area of MFGM research has advanced considerably since the previous edition of this book (Keenan and Dylewski, 1995). Some heretofore unrecognized MFGM proteins have been identified, the sequences of the major proteins have been determined, mostly *via* cDNA sequencing, and a function has been ascribed to some proteins. Much of the knowledge in this area has been reviewed extensively (Mather, 2000). Herein, we will provide a summary of this information but will not include sequences. Appropriate data-bank accession numbers are given for those MFGM proteins that have been sequenced. The major proteins of bovine MFGM, for which the sequence has been determined, are the epithelial mucins MUC1 and MUC15, Xanthine oxidoreductase (XDH), cluster of differentiation 36 (CD36), butyrophilin (BTN), adipophilin (ADPH; this protein is known also as adipocyte differentiation-related protein), periodic acid Schiff glycoprotein 6/7 (PAS6/7), and fatty acid-binding protein (FABP) (Figure 4.8). The nomenclature used herein will be largely that recommended by Mather (2000). This latter article also lists other names used for the major MFGM proteins.

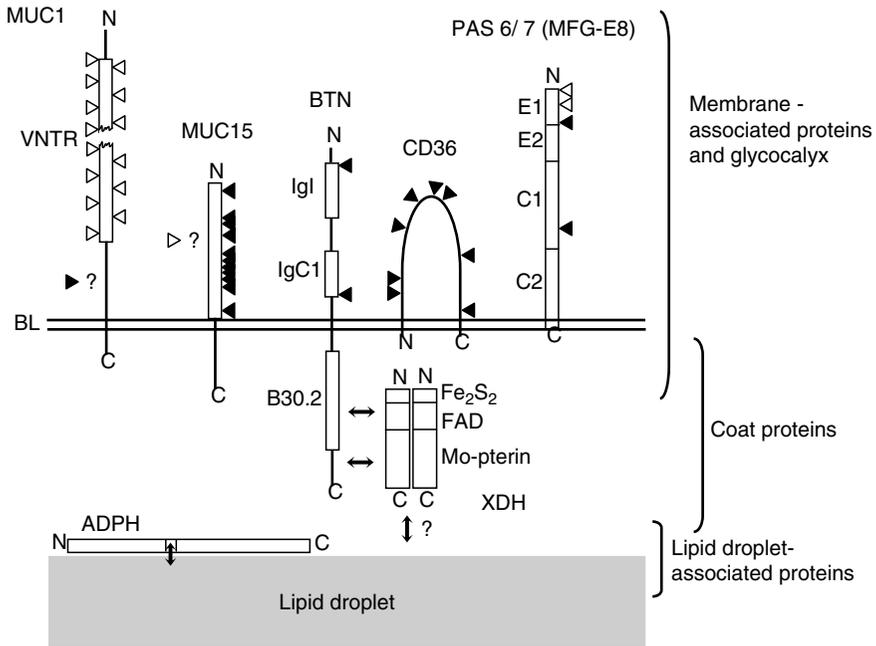
The MFGM of cow, and of most other ruminants, rodent and primate species that have been analyzed, contain one or more heavily-glycosylated mucin-like glycoproteins which stain well with the PAS reagent, variably with a modified silver stain, but poorly or not at all with Coomassie blue. Bovine MFGM contains at least two mucins, MUC1 and MUC15 (formerly PAS III), which have been characterized by biochemical and molecular cloning techniques.

MUC1 mucins have type 1 topologies (i.e., an externally oriented N-terminus and a single transmembrane domain), extensively glycosylated exoplasmic domains, a variable number of tandem repeats in the amino acid sequence, and a short cytoplasmic tail (reviews, Patton *et al.*, 1995; Mather, 2000) (Figure 4.9). During sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), MUC1 migrates with a slower mobility than XDH. In mixed herd milk, MUC1 is seen as a diffuse, smeared band due to allelic polymorphism, although in milk from individual animals it can be resolved as one or two discrete bands. Five alleles have been identified in Holstein cattle in the United States, with an apparent molecular weight ranging from about 160 to 200 kDa (Hens *et al.*, 1995; Huott *et al.*, 1995). Several MUC1 alleles have been identified in other breeds of cattle (for review; see Mather, 2000).



**Figure 4.8.** Polypeptides associated with washed milk fat globules (G), released milk fat globule membranes (M), and with the detergent and high salt-insoluble MFGM coat material. (a) Gel was stained with Coomassie blue. The abbreviations are XDH, xanthine dehydrogenase/oxidoreductase; BTN, butyrophilin; ADPH, adipophilin; PAS 6/7, periodic acid Schiff 6/7; FABP, fatty acid binding protein. (b) Gel was stained with silver. The positions of the major proteins, including MUC1 and CD36, are indicated by arrowheads and the approximate position of MUC15 by a bar (MUC15 is best detected with the PAS reagent, see Mather, 2000). The unlabeled arrowheads in (b) denote the corresponding proteins identified in (a). SDS-PAGE was in 8 to 16% Tris-glycine gels.

Bovine milk MUC1 is present in both cream and skim milk membranes, at an estimated total concentration of up to about 40 mg/L of milk (Mather, 2000). MUC1 has been purified from bovine MFGM and a full-length cDNA described (EMBL accession number **AJ400824**; Pallesen *et al.*, 2001). The complete sequence is available also for MUC1 from human (**J05581**), gibbon (**L41589**), mouse (**M65132**) and other species. While the amino acid sequence predicts a trans-membrane protein, milk MUC1 behaves anomalously as a soluble protein and can be displaced readily, at least in part, from the MFGM (Mather, 2000). Soluble forms of MUC1, lacking the membrane anchor, have been described. In cultured cells, a proteolytic cleavage occurs in the exoplasmic domain, generating a noncovalently associated heterodimer (Ligtenberg *et al.*, 1992). There is some preliminary



**Figure 4.9.** Protein topology and proposed structure of the bovine MFGM. Proteins and their constituent domains are drawn schematically showing the approximate positions of N-linked glycans (closed triangles) and O-linked glycans (open triangles). The number and positions of N-linked glycans associated with MUC1, and of O-linked glycans associated with MUC15 is uncertain. Glycosylation sites in PAS6/7 are shown as a composite of both PAS6 and 7. Integral proteins of the bilayer (BL) include MUC1 with associated variable-number-tandem repeats (VNTRs) (indicated as a break in the sequence), MUC15, CD36 and BTN with associated intermediate (Igl) and constant (IgC1) Ig domains in the exoplasmic domain and B30.2 region in the cytoplasmic domain. PAS6/7 with associated EGF-like repeats (E1 and E2) and factor-V- and VIII-like domains (C1 and C2) may be anchored to the membrane *via* a C-terminal amphipathic alpha-helical domain. Glycans associated with MUC1, MUC15, BTN, CD36 and PAS6/7 and additional glycosaminoglycans (not shown) contribute to the outer glycocalyx of the membrane. The major constituents of the protein coat on the inner surface of the MFGM comprise XDH [shown as a homodimer, with constituent Fe/sulfur clusters ( $Fe_2S_2$ ), flavin-binding domain (FAD) and molybdo-pterin domain (Mo-pterin)], the cytoplasmic tail of BTN and ADPH. ADPH is bound to the lipid droplet surface through a presumptive alpha-helical domain. Double arrowheads indicate interactions between BTN and XDH and between ADPH and the lipid droplet surface discussed in the text. Whether XDH also interacts with the lipid droplet surface is speculative. In molar terms, BTN and XDH are the most abundant membrane-associated proteins. Figure adapted from Figure 4.5b of Mather and Keenan (1998).

evidence that such is the case with bovine MFGM MUC1 (S. Patton and T. W. Keenan, unpublished).

Apparently MUC1 does not play a direct role in milk-fat globule secretion, as MUC1 knock-out mice lactate normally and raise litters successfully (Spicer *et al.*, 1995). This does not preclude an ancillary role for MUC1 in the secretory process or a role in stabilizing the MFGM. There is some evidence that MUC1 plays an immuno-protective role in the suckling neonate by sequestering pathogenic microorganisms (for reviews, see Mather, 2000; Patton, 2001; Peterson *et al.*, 2001).

A second mucin, MUC15, previously called PAS III, in bovine MFGM (Kaetzel *et al.*, 1987; Mather, 2000), has been isolated recently and characterized by biochemical and molecular cloning techniques (**EMBL accession number AJ417816**; Pallesen *et al.*, 2002). The human homologue also was cloned and shares 67% similarity in linear sequence with bovine MUC15. MUC15 was identified originally as a diffuse band of PAS-reactive material with an apparent molecular weight between about 95 to over 100 kDa, following SDS-PAGE (see, for example, Mather, 2000). Typically, this glycoprotein does not stain with Coomassie blue and variably with the silver reagent (position shown by a square bracket in Figure 4.8).

Like MUC1, MUC15 is a type 1 glycoprotein with an extensive extraplasmic domain, a single membrane span and a short cytoplasmic tail (Figure 4.9). The predicted molecular mass of 33.3 kDa is much smaller than expected from the electrophoretic mobility in SDS-polyacrylamide gels, suggesting that a large proportion of the mass of MUC15 is carbohydrate (Pallesen *et al.*, 2002). Both O-linked and N-linked glycans were detected by biochemical methods. Unlike MUC1, there are no tandem repeats in MUC15. The polydisperse character of MUC15 is, therefore, presumably due to the addition of variable amounts of carbohydrate. A splice variant, encoding a soluble form of MUC15, lacking the transmembrane domain (MUC15/S), was described also. MUC15 and MUC15/S, are widely expressed (at least at the RNA level) in many epithelial and non-epithelial cells and are currently of unknown function.

XDH is a major protein of the MFGM, with a monomeric molecular mass of about 155 kDa, estimated by SDS-PAGE (Figure 4.8). The active form of this enzyme is a homodimer. XDH accounts for about 20% of the globule-associated protein (Mather *et al.*, 1977). Upon disruption of the membrane, large amounts of XDH are recovered in the MFGM supernatant, and the remainder is bound tightly to the membrane (Mather *et al.*, 1977; Jarasch *et al.*, 1981). This bound XDH is recovered largely in the high salt and non-ionic detergent-insoluble MFGM coat (Freudenstein *et al.*, 1979). XDH is a widely-studied redox enzyme and has been the subject of numerous reviews (for references, see Mather, 2000). The sequence of XDH

from several species has been determined; in most cases from the cDNA. The sequence of bovine XDH (**Genbank accession number X83508**) is very similar to that of several other mammalian species. The amino acid sequence identity between bovine, rat, mouse, and human enzymes is close to 90% (Mather, 2000).

While XDH plays a key role in purine catabolism, the large amount of this enzyme associated with fat globules suggests a function beyond purine oxidation. As discussed above, XDH may play a role in milk fat globule secretion. Expression of the XDH gene increases during pregnancy and becomes maximal after parturition (McManaman *et al.*, 2002). Recently, direct evidence for a role for XDH in milk fat secretion was obtained. Vorbach *et al.* (2002) generated mice with a targeted disruption in the (*Xdh*) gene. (*Xdh*<sup>-/-</sup>) mice were runted and did not live beyond 6 weeks. (*Xdh*<sup>+/-</sup>) females were healthy and fertile but were unable to maintain lactation; in these animals, the mammary epithelium collapsed, resulting in premature involution. XDH was found to be necessary for enveloping lipid droplets in plasma membrane during secretion. In (*Xdh*<sup>+/-</sup>) mice, mammary epithelial cells became engorged with lipid and secreted lipid globules had a fragmented outer membrane that caused them to aggregate into very large droplets.

Cluster of differentiation 36 (CD36), a protein of 76 to 78 kDa, stains weakly with Coomassie blue but strongly with the PAS reagent and modified silver stain (Figure 4.8). This integral protein of the MFGM is heavily glycosylated. The complete amino acid sequence derived from the cDNA from cloned bovine mammary gland is available (**Genbank accession number X91503**). CD36 is known to have a number of diverse, mostly receptor, functions in the vascular and hemopoietic systems, but none of these functions is such as to suggest any role in milk fat secretion (Mather, 2000). However, there is evidence that human CD36 can associate with *src*-family kinases and function in intracellular signalling (Greenwalt *et al.*, 1992). Given the evidence suggestive of the involvement of kinases/phosphatases in milk-fat secretion (discussed in the section on milk fat-globule secretion), the possible role of CD36 as a modulator of lipid secretion should be explored.

BTN is the most abundant protein of bovine MFGM and has been estimated to account for about 35 to 40% of the total MFGM protein in Holstein milk and for about 20% of MFGM protein in Jersey milk (Mather *et al.*, 1980; Mondy and Keenan, 1993). The name “butyrophilin” is derived from the Greek *butyros* and *philos* meaning an affinity for butterfat (Franken *et al.*, 1981). BTN migrates during SDS-PAGE with an estimated molecular mass of about 66 kDa and stains readily with Coomassie blue and silver (Figure 4.8). The exoplasmic domain contains two N-linked glycans that have been characterized extensively (Sato *et al.*, 1995; Mather, 2000). BTN is

expressed specifically in the mammary gland and is concentrated in the apical plasma membrane and the MFGM. On destabilization of fat globules, most of the BTN remains associated with the membrane. Upon disruption of membrane structure by extraction with chaotropic agents, high salt or non-ionic detergents, much of the BTN remains associated with the insoluble MFGM coat material.

Gene and cDNA sequences for bovine BTN (**Genbank/EMBL accession numbers M35551; Z93323**) and BTN from some other species are available. At least ten related BTN genes have been identified in several species that have limited to broad expression profiles in tissues other than the mammary gland (e.g., Ye *et al.*, 2000; Rhodes *et al.*, 2001). Following the convention of the HUGO Gene Nomenclature Committee, the mammary-specific gene should be denoted *BTN1A1*, to distinguish it from related family members. BTN has an externally oriented N-terminus and a single trans-membrane anchor located close to the middle of the sequence (type 1 topology) (Jack and Mather, 1990; Banghart *et al.*, 1998) (Figure 4.9). BTN and related family members belong to the immunoglobulin (Ig) superfamily of adhesive and receptor proteins. BTN has two Ig-like folds in the N-terminal half of the molecule. In the C-terminal part, there is a B30.2 domain, predicted to comprise two Ig folds (Sato *et al.*, 1995), which may function as a protein-binding domain (Schweiger *et al.*, 1999; Mather, 2000; Niikura *et al.*, 2003; Zhai *et al.*, 2004). As discussed in the section on secretion of milk fat globules, BTN associates with XDH, and probably with other proteins, to form a large complex.

It was long speculated that BTN plays some role in milk fat secretion (Mather and Jack, 1993) and now there is direct evidence for this. Ogg *et al.* (2004) prepared two lines of mice in which either variable amounts of a truncated form of BTN were expressed, or BTN expression was undetectable. Females of both lines were fertile and entered into lactation but had defects in fat globule secretion. In these mice, just like in (*Xdh*<sup>+/-</sup>) mice, secretory cells became engorged with lipids. Lipid droplets were secreted with disrupted membranes and aggregated into large droplets.

ADPH was not identified as a major MFGM protein until recently because it co-migrates with the glycoprotein known as PAS 6/7. ADPH was known previously as adipocyte differentiation-related protein (ADRP) because it is expressed early during adipocyte differentiation and was believed to be expressed only in adipocytes (Jiang and Serrero, 1992). Since then, ADPH has been detected in a large number of tissues and cell types, where it invariably is associated with intracellular lipid droplets (Heid *et al.*, 1996; 1998; Brasaemle *et al.*, 1997).

ADPH migrates as an estimated 52 to 54 kDa polypeptide on SDS-PAGE (Figure 4.8). It remains largely associated with the MFGM, even

though it is not a trans-membrane protein, when globules are destabilized and it is recovered mostly, if not entirely, in the MFGM-associated coat material when membrane structure is destroyed (Heid *et al.*, 1996). ADPH appears to be intermediate in abundance between BTN and XDH in bovine MFGM. This protein probably is not glycosylated (Heid *et al.*, 1996).

The sequence of bovine ADPH was determined from its cDNA (**Genbank accession number AJ011680**); the bovine protein is 87 and 80% identical to human and mouse ADPH, respectively (Nielsen *et al.*, 1999). Surprisingly, ADPH does not have an extensive contiguous sequence of hydrophobic amino acids. About half of the 50 N-terminal residues are nonpolar. McManaman *et al.* (2003) found that the lipid-targeting domain of ADPH is in a presumptive  $\alpha$ -helical region between residues 189 and 205 of the mouse protein (Figure 4.9).

PAS 6/7, the bovine homolog of mouse MFG-E8, has been known by many names (Mather, 2000). This protein migrates as a broad doublet with an estimated molecular mass range of 43 to 58 kDa on SDS-PAGE (Figure 4.8). PAS 6/7 stains both with Coomassie blue and with the PAS reagent. PAS 6/7 is an extrinsic protein of the MFGM and can be largely displaced from the membrane by extraction with buffers containing a high concentration of salt (Mather, 2000). Upon destabilization of fat globules, a high proportion is recovered in the MFGM supernatant fraction.

The complete amino acid sequence derived from cDNA is available for bovine PAS 6/7 (**Genbank accession number X91895**) as well as the homologous protein from several other species (Mather, 2000). PAS 6/7 is the product of a single gene but gives rise to a doublet on SDS-PAGE because of variable glycosylation of an identical polypeptide chain. The protein contains both N-linked and O-linked glycan chains. In the N-terminal region of the protein, there are two epidermal growth factor-like domains, one of which contains the Arg-Gly-Asp adhesive sequence that binds to integrin receptors. PAS 6/7 also contains domains similar to the C1 and C2 domains of blood-clotting factors V and VIII (Figure 4.9). The C2 domain contains putative phospholipid-binding motifs and topological analysis suggests that PAS 6/7 is bound to anionic phospholipids of the MFGM (Mather, 2000). Sequence analysis of the cDNA of the rat homolog of PAS 6/7 provided evidence that this protein may be an acetyltransferase that functions in O-acetylation of ganglioside sialic acids (Ogura *et al.*, 1996), but whether the MFGM protein has such enzymic activity remains to be determined.

PAS 6/7 has no known role in milk-fat globule secretion. The human homologue, lactadherin, binds to rotaviruses and may protect the gastrointestinal tract of the suckling young from infection (Peterson *et al.*, 2001).

The mouse homologue, MFG-E8, plays a direct role in apoptosis as *MFG-E8*<sup>-/-</sup> mice are defective in the phagocytosis of apoptotic cells by macrophages (Hanayama *et al.*, 2004). MFG-E8 is secreted by activated macrophages and binds to phosphatidylserine expressed on the outer surface of apoptotic cells, thus stimulating their subsequent destruction by phagocytosis (Hanayama *et al.*, 2002).

FABP can be identified during SDS-PAGE of the MFGM as a protein that migrates with an estimated molecular weight of 13 kDa (Figure 4.8). Gels containing more than 10% polyacrylamide, or gradient gels, are required to resolve this protein from material that migrates at or near the dye front in gels of lower polyacrylamide concentration (Mather, 2000). This protein stains with Coomassie blue but not with the PAS reagent and, upon destabilization of fat globules, is recovered largely in the MFGM supernatant fraction. FABP was identified originally as a potent inhibitor of the growth of mammary carcinoma cells and was called “mammary-derived growth inhibitor” (Mather, 2000).

There are many tissue-specific or cell-specific forms of FABP; the MFGM form is most likely to be heart FABP, the sequence of which has been determined (**Swiss Protein accession number P10790**). However, it is possible that other forms of FABP also are associated with the MFGM (Mather, 2000). As yet, no functional role has been found for FABP of MFGM.

In addition to the major MFGM proteins, numerous other, quantitatively minor, polypeptides are detectable, especially when SDS gels are stained with silver (Figure 4.8). Two-dimensional separations reveal a complex pattern of minor constituent proteins (see, for example, Mather, 2000). Many of these constituents have yet to be identified in bovine MFGM (however, see Quaranta *et al.*, 2001, Cavaletto *et al.*, 2002 and Wu *et al.*, 2000 for proteomic studies of human and mouse MFGM). Some of these unidentified proteins are likely to be enzymes, as a large number of enzymic activities have been detected in MFGM (Table 4.3). In addition, one would expect that at least some of the proteins associated with the surface of lipid droplets within the cell are present in secreted milk-fat globules. However, the only such protein specifically identified is ADPH. Monomeric GTP-binding proteins associated with the MFGM have been identified by ligand binding but which of the minor silver-stained constituents migrating in the 19 to 25 kDa region correspond to these G-proteins remains to be determined. Recently, some preliminary evidence that a 23 kDa membrane constituent may be the C-terminal MUC1 heterodimeric partner and that a 43 kDa constituent may be actin has been obtained using antibodies for detection; the identity of actin was confirmed by partial sequence analysis (Heid and Keenan, 2005).

#### 4.9. Molecular Organization of the MFGM

The organization of material on the fat globule surface has been the subject of much speculation and experimentation over the past about 90 years. Models of the nature and organization of the fat globule surface material have progressed from it being an adsorbed layer of constituents derived from milk serum to the current view that the MFGM is a true biological membrane. Five groups made seminal contributions leading to our current view of MFGM structure. Bargmann and Knoop (1959) were the first to provide electron micrographs showing that fat globules are secreted from cells by envelopment in apical plasma membrane. The biochemical studies of Morton and colleagues (Morton, 1954; Bailie and Morton, 1958a,b) and of Brunner and colleagues (Dowben *et al.*, 1967) provided evidence that the MFGM contains a number of enzymes typically found in cellular membranes. Patton and Fowkes (1967) showed that the phospholipids of MFGM are like those of cellular membranes and provided a biophysical rationale for the envelopment of lipid droplets in plasma membrane. Patton and Trams (1971) and Mather and Keenan (1975) showed that certain MFGM proteins are disposed asymmetrically with respect to the plane of the lipid bilayer of the membrane.

Our current view of the MFGM is that it is a true bilayer membrane with a dense protein coat 10 to 50 nm thick oriented on the cytoplasmic membrane face (the face contacting the core lipids of the globule) and an innermost layer derived from material that coated the lipid droplets before secretion. The bilayer membrane of the MFGM almost certainly is derived from specialized regions of apical plasma membrane. The dense protein coat is most probably the complex formed from interaction of cytosolic XDH with the cytoplasmic tails of BTN and ADPH and perhaps other proteins of the intracellular lipid droplet surface (Figure 4.9).

Early morphological and biochemical studies showing that there is a distinct asymmetric orientation of the glycans, enzyme-active sites, and proteins of the MFGM have been in part confirmed by molecular biological approaches (Figure 4.9). With respect to the major proteins, it has been established that PAS 6/7 is an externally disposed extrinsic MFGM constituent and that XDH is an internally disposed constituent of the MFGM coat that interacts with the membrane by association with BTN (for review, see Mather and Keenan, 1998). MUC1, MUC15 and BTN are trans-membrane proteins with single membrane-spanning regions and with externally-oriented N-termini. CD36 also is a trans-membrane protein but has short, internally-disposed N-terminal and C-terminal segments and a large, externally-disposed loop. Apparently, ADPH does not occur in plasma membrane

(Heid *et al.*, 1996), so it is likely that this protein originates from the surface of intracellular lipid droplets and interacts with BTN, XDH, or perhaps other proteins or protein complexes on the inner face of the MFGM. Phospholipids and glycosphingolipids are known to be asymmetrically organized in cellular membranes but we have no specific information as to how these constituents are oriented in the MFGM.

#### 4.10. Perspectives

Much of the progress made in this research area during the past 10 years has been in elucidating the sequence and orientation of MFGM proteins and establishing that XDH and BTN are necessary for the normal secretion of milk-fat globules. Data suggestive of factors that regulate the actual milk-fat globule secretory process have also become available. In contrast, there has been little advance in our knowledge of how intracellular precursors of milk lipid globules form and grow prior to secretion. We now have a knowledge base sufficient to allow drawing of diagrams showing the origin, growth, transit, and secretion of milk fat globules (Figure 4.5) but there is a severe lack of understanding of the molecular mechanisms involved in these processes. This research area is ripe for the application of molecular cell biological approaches to answer the many remaining questions.

One major obstacle to molecular studies in the area of milk-fat globule formation and secretion is the lack of a cell line that secretes, or can be induced to secrete, milk fat globules, although some progress in this area has been reported (Rohlfes *et al.*, 1993). Development of such a cell line would be a major advance, in and of itself, and would be an invaluable aid in fostering further research in this area.

#### Acknowledgements

We express our gratitude to all of the colleagues who have worked with us in this research area. Special thanks are due to Stuart Patton for many provocative discussions and suggestions. The research of TWK reported herein has been supported by the National Science Foundation, The National Institutes of Health, the U.S. Department of Agriculture, and by the Virginia Agricultural Experiment Station. The research of IHM reported herein has been supported by the National Science Foundation, the U.S. Department of Agriculture, and by the Maryland Agricultural Experiment Station.

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# Physical Chemistry of Milk Fat Globules

T. Huppertz and A.L. Kelly

## 5.1. Introduction

The presence of fat globules in milk was first reported by Van Leeuwenhoek in 1674, after microscopic analysis of milk placed in a fine capillary tube; since then, the physical and colloidal properties of milk fat globules and their size distribution have been the subject of considerable study. These properties of milk fat globules are responsible for, or contribute to, some of the properties and phenomena observed in liquid dairy products (e.g., the colour and creaming of milk), and are integral to the manufacture and characteristics of many dairy products (e.g., butter and ice cream). Furthermore, the properties of milk fat globules can influence enzymatic processes, such as lipolysis. Finally, milk fat globules can be affected greatly by processes applied to the milk, particularly homogenization, which has significant implications not only for the properties of milk fat globules, but also of casein micelles in milk.

In this Chapter we will describe important aspects of the physical and colloidal chemistry of milk fat globules, in particular recent research in the area, which underpins many of the phenomena described in other chapters of this book. The relevant aspects of processes that affect the stability of fat globules, including storage, homogenization and heating, and resulting interactions with other milk constituents, including caseins and whey proteins, will be reviewed also.

## 5.2. The Nature and Size Distribution of Milk Fat Globules

Fat in milk is present predominantly in spherical droplets ranging from  $<0.2$  to  $>15\ \mu\text{m}$  in diameter; bovine milk typically contains  $>10^{10}$  fat

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

globules per mL. The composition of the fat in the milk fat globules has been discussed in detail in Chapters 1 and 2. Fat globules in milk are naturally emulsified by a complex layer of surface material, the milk fat globule membrane (MFGM), which accounts for 2–6% of the mass of the fat globules (Keenan and Mather, 2002; Chapter 4). The composition of the MFGM (Table 5.1) is closer to that of a cell membrane, from which it largely derives, than to either milk fat or milk serum. Widely-differing compositions of the MFGM, particularly in terms of triglyceride profile, have been reported. Several enzymes are found in the MFGM fraction, including alkaline phosphatase and Xanthine oxidoreductase, which make up a significant portion of the membrane protein, as well as monoglycerides and free fatty acids. For a more detailed description of the secretion of milk fat globules and the structure of the MFGM, the reader is referred to Chapter 4.

One of the most important properties of fat globules in milk is their size, both in terms of mean (average) size, but the range or distribution of sizes and the effects of processes and treatments thereon. A review of the early studies on milk fat globule size was prepared by Campbell (1932). The size distribution of milk fat globules may vary greatly with the analytical method used, giving a certain degree of unreliability to results obtained using some older methods (Walstra *et al.*, 1969). However, in more recent years, methods such as dynamic light scattering (Robin and Paquin, 1991; Dalgleish and Hallett, 1995), low-angle laser light scattering (Muir *et al.*, 1991; Michalski *et al.*, 2001a), Coulter counting (Hillbrick *et al.*, 1998), ultrasound (Miles *et al.*, 1990) and electroacoustics (Wade *et al.*, 1996; Wade and Beattie, 1997), have been applied to obtain accurate and reproducible results. Many of the measurement techniques mentioned generate complex primary data,

**Table 5.1.** Estimated average composition of milk fat globule membrane (adapted from Walstra *et al.*, 1999)

Component	mg/100 g fat globules	mg/m <sup>2</sup> fat surface	% of membrane material
Protein	1800	9.0	70
Phospholipids	650	3.2	25
Cerebrosides	80	0.4	3
Cholesterol	40	0.2	2
Neutral glycerides	+ <sup>a</sup>	+	?
Water	+	+	?
Carotenoids and vitamin A	0.04	$2 \times 10^{-4}$	–
Iron	0.3	$1.5 \times 10^{-3}$	+
Copper	0.01	$5 \times 10^{-5}$	–
<i>Total</i>	>2570	>12.8	100

<sup>a</sup> + indicates component is present but concentration has not been determined precisely.

which must be processed using specific algorithms or programmes to yield useful data for milk fat globule size.

Avoidance of interference of other milk constituents with measurements is also of importance; for example, dissociation of casein micelles by calcium-chelating agents, such as trisodium citrate or ethylenediamine tetraacetic acid (EDTA), may be used to avoid interference of the micelles in particle size measurement, while clusters of fat globules can be disrupted by adding a low level of sodium dodecyl sulphate (SDS).

A plot of the number distribution (i.e., the number of globules per unit volume,  $N$ , in a certain size class, divided by the width of the size class,  $\Delta d$ ), as a function of size,  $d$ , shows three sub-distributions (Walstra, 1969a, 1995): a subclass of 'small particles', comprising  $\sim 80\%$  of the number of particles but only  $\sim 3\%$  of the mass of fat, the main fraction, comprising  $\sim 95\%$  of fat, and a subclass of large globules, comprising  $\sim 2\%$  of the fat. Besides the number distribution, distributions of mass, volume or surface area can also be calculated, by multiplication of the number frequency by mass, volume or surface area, respectively, for each size class. Plotting volume frequency *versus* particle diameter is the most common method of presentation of globule size data (Walstra, 2003).

Several parameters can be used to express the mean size of the milk fat globules. These parameters are derived from the so-called moments of the size distribution function; the  $n$ th moment of the distribution function ( $S_n$ ) is equal to:

$$S_n = \sum d_i^n N_i$$

where  $N_i$  is the number of particles present and  $d_i$  is the particle diameter in size class  $i$ . These moments have no physical meaning, but are particularly useful as auxiliary parameters in the calculation of characteristic numbers of size distribution. Some common parameters characterizing mean globule size are given in Table 5.2, as are means and ranges of such values for bovine milk. The specific surface area of the fat globules,  $A$ , can be derived from the volume surface-weighted mean diameter:

$$A = 6\phi/d_{3,2}$$

where  $\phi$  is the volume fraction of milk fat and  $d_{3,2}$  is volume surface-weighted mean diameter. A typical mean value for  $A$  is  $\sim 2.2 \text{ m}^2/\text{g}$  fat in unhomogenized bovine milk (range  $1.9\text{--}2.5 \text{ m}^2/\text{g}$  fat; Walstra, 1969b).

Average milk fat globule size decreases with advancing stage of lactation (Walstra, 1969a) and is positively correlated with the fat content of the milk (Wiking *et al.*, 2003) and daily fat yield (Wiking *et al.*, 2004).

**Table 5.2.** Parameters describing the size distribution of milk fat globules in unhomogenized bovine milk

	Range ( $\mu\text{m}$ )	Average ( $\mu\text{m}$ )
Number mean diameter: $d_n = d_{1,0} = S_1/S_0$	0.67–1.0	0.81 <sup>a</sup>
Volume mean diameter: $d_v = d_{3,0} = (S_3/S_0)^{1/3}$	1.5–2.1	1.8 <sup>a</sup>
Volume surface-weighted mean diameter: $d_{vs} = d_{3,2} = S_3/S_2$	2.5–4.6	3.34 <sup>a</sup>
Volume moment-weighted mean diameter $d_{vm} = d_{4,3} = S_4/S_3$		3.53 <sup>b</sup>

<sup>a</sup> Walstra (1969a).<sup>b</sup> From Huppertz *et al.* (2003).**Table 5.3.** Fat globule size (volume surface-weighted mean diameter,  $d_{vs}$ ) in milk from various species

Species	$d_{vs}$ ( $\mu\text{m}$ )	Reference
Cow	3.9	Walstra (1969a)
	4.0	Rüegg and Blanc (1981)
	~3.5	Van Boekel and Folkerts (1991)
	5.32	Mehaia (1995)
Goat	3.51	Attaie and Richter (2000)
	4.89	Mehaia (1995)
Camel	2.76	Attaie and Richter (2000)
	4.40	Farah and Rüegg (1991)
Human milk colostrum	4.40	Mehaia (1995)
	1.74	Rüegg and Blanc (1981)
Human milk transitional	1.84	Rüegg and Blanc (1981)
Human milk mature	4.10	Rüegg and Blanc (1981)
Ewe	5.00	Gervilla <i>et al.</i> (2001)
	4.95	Mehaia (1995)

As illustrated in Table 5.3, considerable interspecies differences in milk fat globule size have been reported. Compared to bovine milk,  $d_{3,2}$  is lower for fat globules in caprine (Mehaia, 1995; Attaie and Richter, 2000) and ovine milk (Mehaia, 1995), but is similar in mature human (Rüegg and Blanc, 1981) and camel (Farah and Rüegg, 1991) milk.

Milk fat globule size can be influenced by several treatments applied to milk. Homogenization, as discussed in Section 5.12, is a mechanical treatment classically applied by milk processors to reduce fat globule size and prevent creaming during the storage of liquid milk. Van Boekel and Folkerts (1991) reported that batch heating or indirect ultra-high temperature (UHT) heating of milk at 90–150°C did not influence volume surface-weighted mean diameter  $d_{vs}$ , whereas direct UHT heating reduced

$d_{vs}$  progressively with increasing temperature. Treatment of milk or cream at a high hydrostatic pressure (up to 600 MPa) has little effect on milk fat globule size (Dumay *et al.*, 1996; Gervilla *et al.*, 2001; Huppertz *et al.*, 2003).

### 5.3. Differences in the Composition of Milk Fat Globules

Walstra and Borggreve (1966) reported that, in milk from a single milking of a single cow, considerable differences in refractive index existed between milk fat globules of similar diameter, indicating differences in the composition of the globules. Furthermore, the observation by Walstra (1967) that the fat in a small proportion of the fat globules in milk melts at a temperature considerably higher than the average melting point (37°C) also indicates compositional differences between fat globules.

The composition (i.e., fatty acid composition of the triacylglycerols) of fat globules also varies with globule size. Timmen and Patton (1988) found less C<sub>4:0</sub> – C<sub>10:0</sub> and C<sub>18:0</sub> and more C<sub>18:1</sub> acids in smaller than in larger fat globules. The fatty acid composition of globules also differs with season; the C<sub>18:1</sub> and C<sub>18:2</sub> acid content of milk obtained in winter increases with fat globule size, but the opposite effect is observed in spring milk; in winter, the levels of C<sub>14:0</sub> and C<sub>16:0</sub> acids decrease with fat globule size (Briard *et al.*, 2003). In both spring and winter, there was significantly more C<sub>14:0</sub>, C<sub>16:1</sub> and less C<sub>18:0</sub> acids in small fat globules compared to large globules (Briard *et al.*, 2003). The higher levels of C<sub>18:1</sub> and C<sub>18:2</sub> acids in small than in large globules in spring milk can be explained partially by the fact that the fat globule membrane, which represents a larger proportion of the mass of smaller globules, contains a higher proportion of these fatty acids than bulk fat (Jensen and Nielsen, 1996); however, the larger proportion of C<sub>18:1</sub> and C<sub>18:2</sub> acids in the membrane alone cannot fully explain compositional differences between globules of different size; thus, it may be assumed that their level in the fat core is also higher (Briard *et al.*, 2003). Wiking *et al.* (2004) reported a positive correlation between the average fat globule size in milk and the concentration of C<sub>16:0</sub>, C<sub>16:1</sub>, C<sub>18:0</sub> and C<sub>18:1</sub> acids.

### 5.4. Fat Crystals in Globules

Crystallization of fats (triglycerides) is a complex phenomenon, especially for milk fat, due to its very broad fatty acid composition (see Chapter 1). Principles of crystallization of milk fat have been reviewed extensively elsewhere (Chapter 7; Mulder and Walstra, 1974; Walstra *et al.*, 1995). Whether a quantity of milk fat is present as a continuous mass (e.g., anhydrous milk

fat or butter oil) or in numerous small globules (e.g., as in milk or cream) has a considerable influence on its crystallization behavior. The crystallization status affects many properties of the milk fat globules (e.g., their susceptibility to partial coalescence and their resistance to disruption). Some reasons why crystallization of fat in globules may differ from that in bulk milk fat as proposed by Mulder and Walstra (1974) are:

- Heat dissipation in bulk fat is considerably slower than in milk or cream; this is related to the lower thermal conductivity of bulk fat and, in particular, the fact that bulk fat cannot be agitated efficiently.
- Not all fat globules may contain the catalytic impurities required to start heterogeneous nucleation, so that nuclei would have to form spontaneously in those globules. Söderberg *et al.* (1989) observed that deeper super-cooling was necessary to induce crystallization in milk fat globules than in bulk fat, whereas Lopez *et al.* (2002a) observed that, with decreasing globule size in cream, deeper super-cooling was required for crystallization of milk fat inside the globules.
- The surface layer of the fat globule may act as a catalytic impurity (e.g., when it contains mono-glycerides or di-glycerides with long-chain fatty acid residues); however, there is still some uncertainty as to whether this process actually occurs (see Walstra, 1995). Although concentric layers of apparently crystalline fat have been observed in electron micrographs of freeze-etched or freeze-fractured milk or cream samples (Buchheim, 1970; Henson *et al.*, 1971), these observations could not be confirmed by other microscopy techniques. Noda and Yamamoto (1994) reported that it is thermodynamically more favorable for fat crystals to be located at the oil/water interface, rather than in the interior of the droplet, which may explain the presence of fat crystals at the membrane.
- The composition of bulk fat is uniform, but differences from globule to globule are known to occur (see Section 5.3); consequently, considerable differences may occur in the final melting point of the fat between different globules.
- The dispersed state has a considerable effect on fat crystal polymorphism. Lopez *et al.* (2000, 2001c) observed that crystallization in milk fat globules is more disordered than in bulk fat. On slow cooling, milk fat crystallizes in the  $\alpha$  form in cream (Lopez *et al.*, 2001a), whereas in anhydrous milk fat, it crystallizes first in the  $\beta'$  form and then in the  $\alpha$  form (Lopez *et al.*, 2001b). Rapid cooling of cream or anhydrous milk fat from 60 to 4°C leads to the formation of  $\alpha$  crystalline structures, which transformed into  $\beta'$  structures

rapidly in anhydrous milk fat but more slowly in cream. On prolonged storage, these crystal structures evolve further, leading to the co-existence of  $\alpha$ ,  $\beta'$  and  $\beta$  structures (Lopez *et al.*, 2002b). Furthermore, Lopez *et al.* (2002a) observed a greater disorder and smaller size of the fat crystals in milk fat globules as the size of the globules decreased.

Crystallization of milk fat in globules is also influenced by exposure to high hydrostatic pressure. High pressure (HP) treatment at 100–500 MPa at 23°C induces crystallization of fat within the droplets and crystallization proceeds further during storage at 23°C (Buchheim and Abou el-Nour, 1992; Buchheim *et al.*, 1996). Acceleration of crystallization of milk fat by HP treatment is due to a shift of the solid/liquid transition temperature towards a higher value (Frede and Buchheim, 2000). HP-induced crystallization of milk fat was strongly delayed by a reduction in fat globule size (Buchheim *et al.*, 1996). These HP-induced changes in the crystallization behavior of globular milk fat may offer opportunities to overcome the necessity for super-cooling to obtain a particular level of crystalline fat.

## 5.5. Colloidal Interactions

Colloidal interactions form the basis of many of the properties of emulsions, as well as the changes observed in emulsions over time; such interactions govern whether droplets remain as separate entities or aggregate. In this section, a brief overview of the predominant colloidal interactions of importance for the stability of emulsions of milk fat globules is given.

The interactions between two emulsion droplets can be described in terms of the interaction energy, or inter-droplet pair potential,  $w(h)$ , which is the energy required to bring two emulsion droplets from an infinite distance apart to a surface-to-surface separation distance,  $h$  (McClements, 1999):

$$w(h) = w_{\text{attractive}}(h) + w_{\text{repulsive}}(h)$$

If attractive forces dominate at all separations,  $w(h)$  is always positive, and the interaction energy (i.e., the free energy needed to bring two droplets from an infinite distance closer together) will be negative, and the droplets will tend to aggregate. Conversely, if repulsive forces dominate at all separations, and the positive interaction energy is several times larger than the average kinetic energy involved in the encounter of two particles by Brownian motion, droplets tend to remain as individual entities. In many cases, however,  $w(h)$  is neither positive or negative over the entire distance  $h$ .

The classical DLVO (Derjaguin-Landau-Verwey-Overbeek) theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) states that the stability of a colloidal system essentially depends on two independent interactions between colloidal particles: van der Waals attractions and electrostatic repulsion:

$$w(h) = w_{\text{van der Waals}}(h) + w_{\text{electrostatic}}(h)$$

*Van der Waals forces* are attractive forces which act between all molecules; they arise from the attraction between orientationally polarized molecules. Their strength decreases with droplet separation, increases with droplet size and depends on the physical properties of the droplets and the surrounding medium and on the thickness and composition of the absorbed emulsifier layer (Bergensstahl and Claesson, 1997; Friberg, 1997; McClements, 1999).

*Electrostatic interactions* occur between molecules that contain a permanent electrical charge. The approach of two identically charged surfaces leads to an increase in the counter-ion concentration between the surfaces, which generates a repulsive force, as a result of increased osmotic pressure (Dickinson and Stainsby, 1988; Bergensstahl and Claesson, 1990, 1997). The surface charge, as estimated by the zeta-potential, is  $\sim -13$  to  $-14$  mV for unhomogenized milk fat globules (Jack and Dahle, 1937; Payens, 1963, 1964; Michalski *et al.*, 2001b) and  $\sim -20$  mV after homogenization (Wade and Beattie, 1997; Michalski *et al.*, 2001b). Dalgleish (1984) reported slightly lower values for the zeta-potential (i.e.,  $-10$  mV for unhomogenized and  $-13$  to  $-17$  mV for homogenized milk fat globules). The overlap of electric double layers will cause a local increase in potential, implying that work must be performed to bring particles closer together.

Thus, according to the DLVO theory, aggregation of milk fat globules should occur if the van der Waals attraction is larger than the electrostatic repulsion. However, calculation of these forces for milk and application of the data to the DLVO theory results in a negative interaction energy at all distances (Walstra, 1995), so that immediate aggregation of milk fat globules should be observed. Aggregation of fat globules, however, does not occur, even when electrostatic interactions are minimal. Thus, there must be a second repulsive force acting (i.e., steric repulsion); the DLVO theory may thus be extended to:

$$w(h) = w_{\text{van der Waals}}(h) + w_{\text{electrostatic}}(h) + w_{\text{steric}}(h)$$

*Repulsive steric forces* are encountered when the outer segments of two polymer-covered surfaces begin to overlap. These interactions usually lead to a repulsive force due to the unfavorable reduction in entropy associated

with confining the chains between surfaces (Tadros and Vincent, 1983; Isrealachvili, 1992; Walstra, 1996, 2003). In the case of milk fat globules, steric repulsion is provided by glycoproteins in the milk fat globule membrane, which have highly hydrophilic moieties protruding from the globule surface. Hydrolysis of these glycoproteins by papain causes aggregation of milk fat globules (Shimizu *et al.*, 1980).

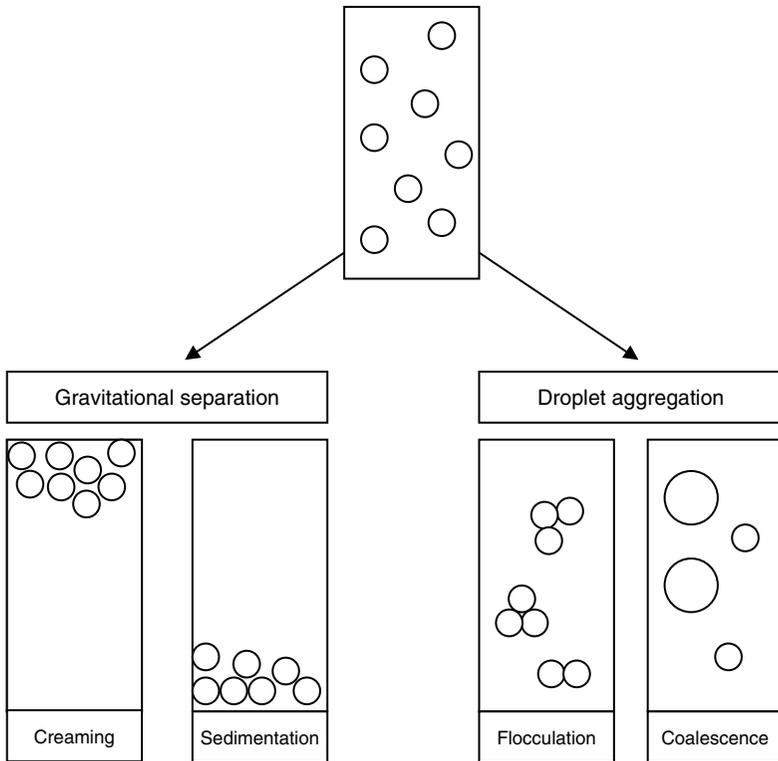
## 5.6. Physical Instability of Emulsions

The stability of an emulsion denotes its ability to resist changes in its properties over time (i.e., higher emulsion stability implies slower change in emulsion properties). When considering the stability of an emulsion, it is of major importance to distinguish between thermodynamic stability and kinetic stability. Thermodynamics predict whether or not a process will occur, whereas kinetics predict the rate of the process, if it does occur. All food emulsions are thermodynamically unstable and thus will break down if left long enough.

Instability of an emulsion may be physical or chemical in nature. Chemical instability, which results in an alteration in the chemical structure of the lipid molecules due to oxidation or hydrolysis (McClements, 1999), will not be considered in this chapter; for more information, the reader is referred to Chapters 11 and 12. Physical instability results in an alteration in the spatial distribution or structural organization of the globules (i.e., the dispersed phase of the emulsion). A number of important mechanisms responsible for the physical instability of emulsions, as depicted in Figure 5.1, can be divided into two categories: gravitational separation and droplet aggregation.

*Gravitational separation* involves the movement of emulsion droplets due to the fact that they differ in density from the surrounding liquid. If the droplets have a lower density than the surrounding medium, they tend to move upwards, a process referred to as *creaming*. Conversely, droplets or particles that have a density higher than the surrounding medium tend to move downwards under the influence of a gravitational force (i.e., *sedimentation*). Creaming of milk will be discussed in more detail in Section 5.7.

*Droplet aggregation* is said to occur when droplets stay together for a time much longer than they would in the absence of colloidal interactions, (i.e., than can be accounted for by collisions due to Brownian motion) (Walstra, 2003). Mechanisms responsible for the physical instability of droplets through aggregation are flocculation, coalescence or partial coalescence.



**Figure 5.1.** Schematic overview of types of instability of emulsions.

- *Flocculation* of droplets is defined as the aggregation of droplets to give three-dimensional floccules, wherein the droplets remain as individual entities (Tadros and Vincent, 1983). Flocculation can be distinguished from coagulation by the fact that the former denotes weak, reversible interactions, whereas the latter denotes strong, and often irreversible, interactions (Walstra, 2003). Flocculation occurs as a result of collisions; the extent of flocculation is determined by both the total number of droplet collisions per unit time per unit emulsion volume and the likelihood that an encounter between droplets will lead to aggregation. The most effective way to control the rate and extent of flocculation is by regulating the colloidal interactions between the droplets.
- *Coalescence* is the process in which two or more fat globules merge to form one larger spherical fat globule through the rupture of the liquid film between emulsion droplets. It is the principal mechanism

by which an emulsion moves towards its thermodynamically-stable state, through a decrease in free energy as a result of the decrease in contact area between the oil and water phases (Tadros and Vincent, 1983; McClements, 1999; Walstra, 1996, 2003). Coalescence of milk fat globules will be discussed in more detail in Section 5.9.

- *Partial coalescence* involves the formation of anisometrically shaped conglomerates of droplets due to the fact that true coalescence is prevented (e.g., because the globules contain a network of crystalline fat) (Walstra, 1996, 2003; McClements, 1999). The ultimate driving force for partial coalescence is a decrease in interfacial free energy, although other processes are also involved (Walstra, 2003). Partial coalescence of milk fat globules will be discussed in more detail in Section 5.9.

## 5.7. Separation of Milk

Because milk fat has a lower density than milk plasma, it tends to rise under the influence of a gravitational or centrifugal force. For perfect spheres, the rate of rise,  $v$ , is given by Stokes' Law:

$$v = a(\rho_p - \rho_f)d^2/18\eta_p$$

where  $a$  is the acceleration due to gravitational or centrifugal force,  $\rho_p$  is the mass density of the plasma,  $\rho_f$  is the mass density of the fat,  $d$  is the diameter of the fat globule and  $\eta_p$  is the viscosity of the plasma. For gravity creaming,  $a = g \approx 9.8 \text{ m/s}^2$ . For creaming under centrifugal force,  $a = R^2\omega$  where  $R$  is the effective centrifugal radius and  $\omega$  is the angular velocity ( $= 2\pi n/60$ , where  $n$  is the number of revolutions per minute).

To predict  $v$  correctly, several prerequisites must be met (Mulder and Walstra, 1974; Walstra and Oortwijn, 1975; Walstra, 1995), most notably:

- Globules must be perfect and homogeneous spheres;
- Other particles in the plasma must be considerably smaller than the fat globules;
- Brownian motion must be small compared to the rate of rise;
- Counter-flow of liquid due to globule movement must be negligible;
- Mutual interaction between globules must be absent.

Troy and Sharp (1928) found that, in milk highly diluted with milk plasma, the rise of individual milk fat globules, as well as roughly spherical clusters of milk fat globules, correlated well with Stokes' law. However, Walstra and Oortwijn (1975) observed that the rate of rise of fat globules in undiluted milk systems under the influence of gravity was lower than

predicted by Stokes' law, in particular for milk of high fat content or containing small fat globules.

The creaming rate (defined as the proportion of the fat arriving in the cream layer per unit time) is proportional to the creaming parameter,  $H$  (Walstra and Oortwijn, 1975):

$$H = S_5/S_3 = \sum N_i d_i^5 / \sum N_i d_i^3$$

This parameter shows a linear relationship with the creaming rate if the effect of aggregation of the globules is excluded (Rüegg and Blanc, 1981); it can be seen that larger globules in particular affect  $H$ , and thus the creaming rate.

The presence of clusters of fat globules affects creaming considerably. Such clusters will rise faster than the individual globules because of their larger size. Clusters may be formed due to cold agglutination (see Section 5.8) or due to inefficient homogenization (i.e., formation of homogenization clusters, see Section 5.13). Also, small clusters of fat globules may be formed during sterilization of heat-evaporated milk at the onset of heat-induced coagulation (Schmidt *et al.*, 1971).

The separation of milk can be accelerated significantly by application of a centrifugal force, which is the principle of separation (skimming) of milk in industrial practice; the design of a separator is depicted in Figure 5.2. The objective of centrifugal separation is to achieve the lowest possible fat content in the skimmed milk, while removing the fat as a greatly (~tenfold) concentrated cream phase. With the exception of high-fat products, Stokes' law can be applied rather accurately to the rate of rise of milk fat globules in a centrifugal field. Centrifugal separation is more efficient at an elevated temperature, as the factor  $(\rho_p - \rho_f)/\eta_p$  increases more than tenfold in a linear fashion over the temperature range 0–80°C (Mulder and Walstra, 1974). The fat content of the skimmed milk depends on the proportion of the fat in very small globules (e.g., < 1 µm), which are the most difficult to separate, and the level of non-globular fat.

## 5.8. Cold Agglutination

When bovine milk is stored in the cold under quiescent conditions, a cream layer will form due to the rise of milk fat globules. However, the rate of rise of the milk fat globules is considerably faster than can be accounted for by Stokes' law for individual globules (Troy and Sharp, 1928). This is due to the fact that milk fat globules tend to rise in large clusters, which rise at a considerably higher rate than individual globules. Merthens (1933b) reported that addition of colostrum to milk enhanced creaming



**Figure 5.2.** Principle of operation of a centrifugal milk separator. Milk enters at the bottom of the unit and separates into heavier skim (dark grey) and lighter cream (pale grey) fractions which are recovered at the top of the separator (Reproduced with permission from *Dairy Processing Handbook*, Tetra Pak Processing Systems AB, Lund, Sweden, 1995).

considerably, suggesting that one or more agents enriched in colostrum promoted creaming. Detailed studies, including an extensive survey of older work in this area, were reviewed by Dunkley and Sommer (1944). The clustering of milk fat globules during cold storage markedly resembles the agglutination of bacteria or red blood cells, due to the action of the

immunoglobulin IgM, in terms of dependence on pH, concentration and valency of cations. Hence, the clustering of milk fat globules in the cold is referred to as cold agglutination.

In terms of understanding the mechanism for cold agglutination of milk fat globules, two of the most important phenomena are the 'Merthens effect' and the 'Samuelson effect'. Merthens (1933a) observed that milk reconstituted from homogenized skim milk and unhomogenized cream has poor creaming ability ("Merthens effect"). It was proposed initially that this is due to denaturation of the agglutinin on homogenization, but Koops *et al.* (1966) showed that this was not the case. Samuelson *et al.* (1954) showed that two components are required for cold agglutination: a homogenization-labile component and a heat-labile component ("Samuelson effect"). Homogenization at a pressure as low as 1 MPa, or even mild shearing, impairs the agglutinating tendency of skimmed milk (Walstra, 1980). The globulin fraction of milk, implicated by many early investigators as the agglutinin, associates with the milk fat globules, particularly in the cold, and is not homogenization-labile (Payens, 1964). Subsequent studies (Payens, 1964, 1968; Payens *et al.*, 1965; Gammack and Gupta, 1967; Payens and Both, 1970; Stadhouders and Hup, 1970) identified immunoglobulin M (IgM) as the heat-labile agglutinin in the globulin fraction of milk. Gammack and Gupta (1970) showed that lipoprotein particles in the aqueous phase are a prerequisite for the rapid creaming of milk, which supports the earlier observations by Hansson (1949) that creaming of milk is enhanced by the addition of phospholipids.

Euber and Brunner (1984) proposed a mechanism for cold agglutination which involves three components: (1) the milk fat globules, (2) IgM, the heat-labile component, which functions as a cold agglutinin; and (3) the so-called skim milk membrane (SMM), the homogenization-labile component, consisting of lipoprotein particles present in the aqueous phase of milk. Euber and Brunner (1984) suggested that these components interact through specific carbohydrate moieties. IgM can interact with both SMM and the fat globules, whereas SMM interacts with IgM only. Fat globules can be clustered to a limited extent by IgM alone, but clustering is considerably more extensive in the presence of SMM, which acts as a cross-linking agent. Environmental factors that affect the uptake of IgM by fat globules or SMM include ionic strength, dielectric constant, pH and the temperature of the suspending medium (Euber and Brunner, 1984).

Cold agglutination is influenced also by processing conditions. Agitation of milk during cold storage impairs creaming, but heating milk to 40–50°C normally restores the creaming capacity of the milk on cold storage (Merthens, 1933a). Heating milk at a higher temperature, up to ~62°C,

improves the creaming capacity, relative to that of raw milk (Rowland, 1937). A similar increase in creaming capacity was observed after high pressure treatment at 100–250 MPa by Huppertz *et al.* (2003), who showed that clusters of milk fat globules formed on cold storage of milk treated at 200 MPa were larger than those formed in unpressurized milk. However, the exact mechanism for heat-induced or HP-induced increases in creaming of milk has not yet been described.

Heating milk at a temperature  $>62^{\circ}\text{C}$  (Orla-Jensen *et al.*, 1929; Rowland, 1937), or treating it at a pressure  $\geq 400$  MPa (Huppertz *et al.*, 2003), impairs the rate of creaming of milk fat globules. Huppertz *et al.* (2003) showed that clustering of milk fat globules on cold storage did not occur in milk treated at 600 MPa. Thermal or high pressure-induced inhibition of cold agglutination is probably the result of denaturation of IgM. Heat-induced interactions of caseins or whey proteins with the MFGM may also prevent cold agglutination (Van Boekel and Walstra, 1995). Addition of colostral globulin to heated milk restores its creaming capacity (Keynon and Jenness, 1958).

Clustering of milk fat globules in the cold, followed by rapid creaming, is not a universal phenomenon. As described earlier, it occurs in bovine milk, but not, or to a considerably lower extent, in caprine (Jenness and Parkash, 1971), ovine (Fahmi *et al.*, 1956) buffalo (Fahmi, 1951; Abo-Elnaga, 1966; Wahba *et al.*, 1977; Ismail *et al.*, 1972), camel (Farah and Rüegg, 1991) or carabao (Gonzales-Janolino, 1968a) milk. This has been related to the fact that clustering of milk fat globules does not occur in the milks from these species.

Jenness and Parkash (1971) showed that milk reconstituted from caprine cream and bovine skim milk creams rapidly, whereas milk reconstituted from bovine cream and caprine skim milk shows a very low level of creaming. Similar results were observed on reconstituting milk cream and skimmed milk from bovine and camel milk, respectively (Farah and Rüegg, 1991). The poor creaming properties of buffalo milk were attributed to its poor clustering ability (Abo-Elnaga *et al.*, 1966). Addition of euglobulin, isolated from buffalo colostrum, considerably increased the creaming capacity of buffalo milk (Wahba *et al.*, 1977). Gonzales-Janolino (1968b) observed poor creaming of mixtures of cows' cream and skimmed carabao milk, whereas a mixture of agglutinin-rich bovine skimmed milk and carabao's cream creamed extensively. Further experiments showed that carabao milk lacks the homogenization-labile component (Gonzales-Janolino, 1968b). Thus, it is apparent that cold agglutination of fat globules in milk is highly dependent on the species of origin.

## 5.9. Coalescence and Partial Coalescence

When two or more emulsion droplets come into contact, a thin film of the liquid continuous phase forms between them. Coalescence is the process whereby liquid droplets merge to form a single larger droplet as a result of the rupture of both this liquid film and the interfacial membrane of the droplets. Coalescence moves an emulsion towards a thermodynamically stable state, because it reduces the contact area between the phases (Tadros and Vincent, 1983; Walstra, 1996; McClements, 1999).

The current state of understanding of coalescence is unsatisfactory because of the number of variables involved and the fact that some fundamental problems have not been resolved fully (Walstra, 2003), but some general understanding has been developed. The susceptibility of droplets to coalescence is determined by the nature of the forces that act on and between the droplets and the resistance of the droplet membrane to rupture. Coalescence may be induced by collisions or by prolonged contact between the emulsion droplets. Collision-induced coalescence can be due to movement of the droplets by Brownian motion, gravity or applied mechanical forces. Coalescence induced by prolonged contact occurs spontaneously after the droplets have been in contact for a sufficient period (e.g., in emulsions, which contain flocculated droplets or droplets that have accumulated at the top or bottom of the emulsion due to gravitational separation). The probability of film rupture is greater if the interfacial tension,  $\gamma$ , is small, and if the colloidal repulsion between the droplets is stronger (Walstra, 2003). Furthermore, susceptibility to coalescence increases with droplet size.

Shimizu *et al.* (1980) reported that removal of the polar head of phospholipids in the milk fat globule membrane by phospholipase C results in oiling-off; thus, it appears that the polar head of the phospholipids plays an important role in the stability of milk fat globules against coalescence.

Indirect UHT treatment can cause aggregation of fat globules, due to partial heat coagulation; direct UHT treatment, which involves greater turbulence and flash boiling, does not cause aggregation (Melsen and Walstra, 1989). Mulder and Walstra (1974) reported that coalescence of fat globules in cream may occur during treatment in a heat exchanger, but Van Boekel and Folkerts (1991) could not confirm this for direct or indirect UHT treatment of unhomogenized milk. Streuper and Van Hooijdonk (1986) observed coalescence on UHT treatment of milk, but only if back-pressure in the apparatus allowed some boiling of the liquid on cooling.

Whereas true coalescence is of limited importance in the case of milk and dairy products, partial coalescence is of far greater importance, in particular in the preparation of products such as whipped cream, butter,

and ice cream. Partial coalescence occurs when two or more partially crystalline emulsion droplets come into contact. A fat crystal protruding from a globule may pierce the film between close globules, which leads to conjunction of the globules, resulting in the formation of an irregularly-shaped aggregate (Van Boekel and Walstra, 1981). The aggregate partially retains the shape of the globules from which it was formed, because the fat crystal network within the droplets prevents complete merger. Partial coalescence differs from true coalescence in that it tends to be much faster and that, due to the formation of irregular aggregates or clumps, it increases the effective volume fraction of the dispersed phase. Prerequisites for partial coalescence include the presence of a network of fat crystals in the globules (Boode *et al.*, 1993) and that the fat crystals are located at the oil-water interface; if fat crystals are totally wetted by either the oil or water phase, they do not affect emulsion stability (Boode and Walstra, 1993).

In the case of milk fat globules, partial coalescence can lead to the formation of irregularly-shaped granules (e.g., butter clumps), or the formation of a continuous network (e.g., whipped cream or ice cream). Walstra *et al.* (1999) reported that the following factors affect the rate of partial coalescence in milk:

- *Application of a velocity gradient* or shear rate increases the rate of collision between fat globules and presses globules closer together, thus enhancing the possibility of a protruding crystal bridging the gap between globules. However, above a certain velocity, the rate of partial coalescence decreases (Boode *et al.*, 1993).
- An increased *fat content* increases the rate of clumping.
- The *proportion of solid fat* is crucial. Partial coalescence can not occur if there are no fat crystals, but if there is too much solid fat, there may not be enough liquid fat to hold globules together (Boode *et al.*, 1993).
- *Fat globule size* also influences the extent of partial coalescence. Larger globules are less stable against partial coalescence, due to the fact they have larger fat crystals and the probability of a crystal sticking out far enough is thus higher.
- The *surface layer on the globules* plays an important role. Natural fat globules are reasonably stable, but the presence of a surface layer of protein (e.g., after homogenization or recombination) increases the stability of the globules considerably, through colloidal repulsion.

Partial coalescence is probably also involved in a defect in unhomogenized milk that has not been kept at a sufficiently low temperature, referred to as “bitty cream” or “broken cream.” Bacterial phospholipases can hydrolyse

up to 60% of the phospholipids in the milk fat globule membrane (O'Mahony and Shipe, 1972), making the globules more susceptible to partial coalescence, which leads to the formation of large particles of cream floating in the milk (Stone, 1952a,b; Stone and Rowlands, 1952; Labots and Gale-sloot, 1959). In market milk, the bitty cream defect has been largely eliminated by homogenization.

## 5.10. Rebodying

The term “rebodying” is often used for the phenomenon whereby cooled cream, when warmed (e.g., to 30°C) and subsequently re-cooled, becomes more viscous, or even (at a sufficiently high fat content) solid-like (Hoffmann, 1999). Rebodying is caused by partial coalescence (Oortwijn and Walstra, 1982b), and occurs in unhomogenized or weakly-homogenized systems, in which much of the natural MFGM is retained. The extent and nature of the rebodding phenomenon depends on the rate of cooling (which determines whether the number [fast cooling] or size [slow cooling] of the fat crystals increases), and the temperature history of the cream, particularly cycling through higher and lower temperatures. Repeated rebodding can cause fat separation. Warming cream that has undergone rebodding to 30°C, increases fat globule size, probably as a result of full coalescence of globules that are already in the partially-coalesced state (Oortwijn and Walstra, 1982b).

In UHT-treated whipping cream, rebodding results in clumping of fat or the formation of cream plugs (small lumps of partially solidified fatty material), which cannot be redispersed in the product by gentle shaking. The fat droplets in the cream plug are aggregated and partially coalesced. Streuper and Van Hooijdonk (1986) reported that the firmness of the plug in UHT-treated cream increased with increasing rate of cooling of the cream. The formation of a cream plug in heat-treated unhomogenized cream can be prevented completely by addition of carrageenan, in combination with an undefined protein-fat powder (Precht *et al.*, 1987). Dickinson *et al.* (1989) introduced the term “cohesive cream” to describe a concentrated emulsion layer in which the flocculated oil or fat droplets have become compressed into a coherent structure that cannot be redispersed by mild agitation; these authors also reported that the formation of cohesive cream in liqueurs is enhanced at a low pH value, a high calcium content or a low level of caseinate emulsifier, as well as by temperature fluctuations during storage.

Recent studies in model creams have further clarified the possible mechanism for rebodding. For an increase in viscosity to occur on re-cooling, it is necessary that, after the warming step, <10% of the fat remains

solid; if all fat is melted, rebodilying does not occur (Boode *et al.*, 1991; Noda and Yamamoto, 1994; Mutoh *et al.*, 2001). Sugimoto *et al.* (2001) observed that the increase in viscosity on re-cooling warmed cream is accompanied by a substantial increase in the concentration of protein on the fat globule surface and proposed the following mechanism for an increase in viscosity on re-cooling warmed cream. At a critical level of solid fat (< 10%) in cream, fat crystals approach the oil droplet surface, which causes conformational changes in the proteins adsorbed at the oil-droplet surface. A rapid decrease in the fluidity of triacylglycerols on cooling causes further changes in the conformation and charge of the surface proteins, which leads to attraction between serum proteins and those on the surface; this results in an increase in viscosity and solidification of the cream. However, further studies are necessary to establish if this mechanism also applies to dairy cream.

### 5.11. Factors that Affect the Surface Layers of Fat Globules in Milk and Cream

The surface layers of the fat globules in milk are affected by various treatments. Effects of homogenization are described in Sections 5.13 and 5.14; however, other treatments such as cooling and heating, as well as environmental conditions, also influence the surface layers of milk fat globules and are described in this section.

Cooling of milk induces the release of up to 15% of phospholipids from the MFGM (Koops and Tarassuk, 1959; Baumrucker and Keenan, 1973), resulting in an increase in the phospholipid content of the milk plasma (Patton *et al.*, 1980). Cooling also causes transfer of Xanthine oxidoreductase from the fat to the plasma phase and results in the reversible adsorption of the cryoglobulins onto the fat globules (Mulder and Walstra, 1974). Furthermore, cooling induces the migration of copper from the milk fat globules to the milk plasma (Mulder and Walstra, 1974). Freezing and subsequent thawing cause considerable clumping of milk fat globules, particularly in cream, primarily caused by pressure differences in the frozen products developing due to the different expansion coefficients of ice and fat (Mulder and Walstra, 1974).

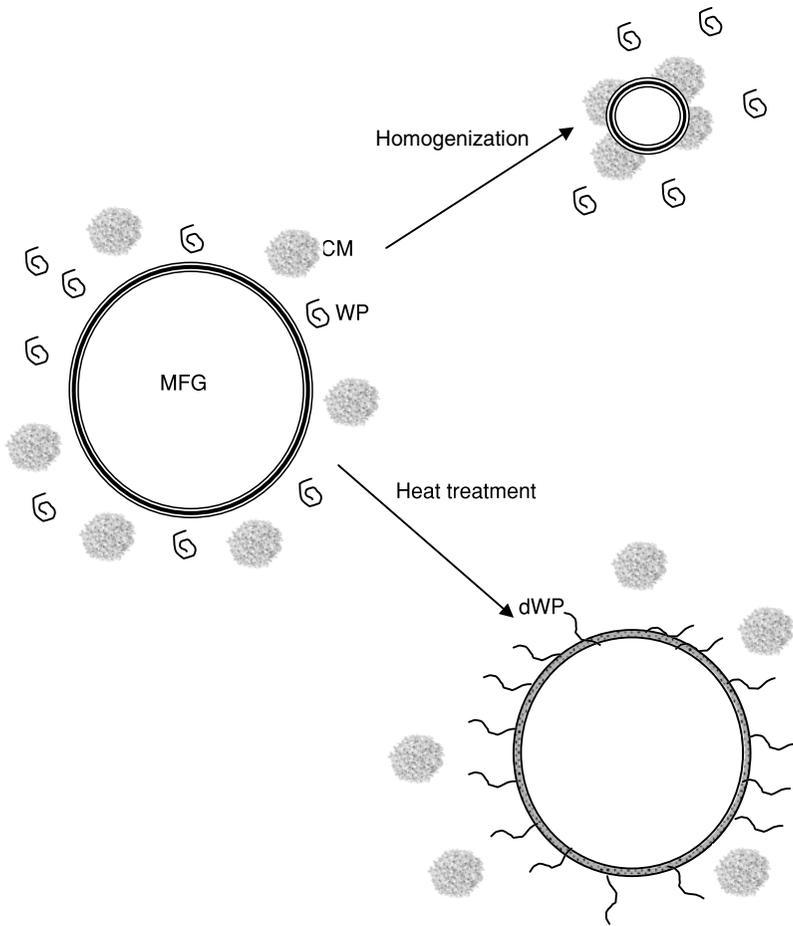
Heat treatment can also affect the composition of the MFGM. The amount of protein associated with the fat globules increases on heating; the newly-bound protein is largely denatured whey protein, particularly  $\beta$ -lactoglobulin (Dalgleish and Banks, 1991; Corredig and Dalgleish, 1998). Interactions of whey proteins with the MFGM probably occur primarily *via* sulphhydryl-disulphide interchange reactions (Kim and Jimenez-Flores, 1995; Lee and Sherbon, 2002). Heating can also result in the formation of high

molecular weight protein complexes between Xanthine oxidoreductase, butyrophilin and denatured whey proteins (Ye *et al.*, 2002); the kinetics of such reactions were reported by Ye *et al.* (2004). If milk is heated prior to homogenization, less whey protein is incorporated into the MFGM than if the order of these steps is reversed (Sharma and Dalgleish, 1994). The association of denatured whey proteins, principally  $\beta$ -lactoglobulin, with the MFGM increases its protein content and reduces that of lipids proportionately. Membrane glycoproteins, such as PAS-6 and 7, become less evident on electrophoretic (SDS-PAGE) analysis following heat treatment (Houlihan *et al.*, 1992; Iametti *et al.*, 1997; Lee and Sherbon, 2002), which may be due to mechanical damage to the globules caused by pumping and circulation through the pasteurizer (Iametti *et al.*, 1997) or to their displacement by denatured whey proteins during heating (Houlihan *et al.*, 1992), although the exact mechanism is thus far unknown (Lee and Sherbon, 2002). The effects of heat treatment and homogenization on fat globules are compared schematically in Figure 5.3. Heat treatment can also result in the release of H<sub>2</sub>S from the globules and transfer of copper from plasma to globules. (Mulder and Walstra, 1974). Heating of milk can also reduce the triacylglycerol content of the MFGM (Houlihan *et al.*, 1992) but conflicting results have been published on the effect of heat treatment on the phospholipid content of the MFGM. Koops and Tarassuk (1959) and Greenbank and Pallansch (1961) observed a reduction in the MFGM phospholipids content on heating, but Houlihan *et al.* (1992) reported that heat treatment did not influence this parameter. In a system comprising milk fat globules in simulated milk ultrafiltrate, warming to 45–50°C for 10 min resulted in the loss of up to 50% of total protein from the MFGM, perhaps due to the melting of the lipid phase and subsequent rearrangement of the globule surface (Ye *et al.*, 2002).

Because concentration of milk by thermal evaporation can also damage the MFGM, and drying can damage the MFGM considerably, milk to be used for evaporation or drying is usually homogenized, to strengthen the globule membranes by binding of caseins. Furthermore, contact with air bubbles can change the MFGM, which has important implications for products such as ice cream and whipping cream, as discussed in Chapters 10 and 13.

## 5.12. Disruption of Globules

Fat globules are relatively fragile, particularly when the fat is liquid, and can be disrupted readily by a number of conditions experienced in dairy processing operations. Shearing, cavitation or turbulence, in particular, can damage the MFGM and cause physical rupture and sub-division of the globules. Rupture occurs when droplets are deformed beyond a critical value for longer



**Figure 5.3.** Schematic illustration of the relative effects of heating and homogenization on fat globules in milk. MFG = milk fat globule, CM = casein micelle, WP = whey protein, dWP = denatured whey protein.

than a critical time. Resistance to deformation is related to the Laplace pressure and the ratio of the viscosity of the fat to that of the plasma. The Laplace pressure refers to the difference between the pressures at the concave and convex sides of a curved interface of two fluids. For a spherical droplet, the Laplace pressure,  $P_L$ , can be expressed as (Walstra, 2003):

$$P_L = 2\gamma/R$$

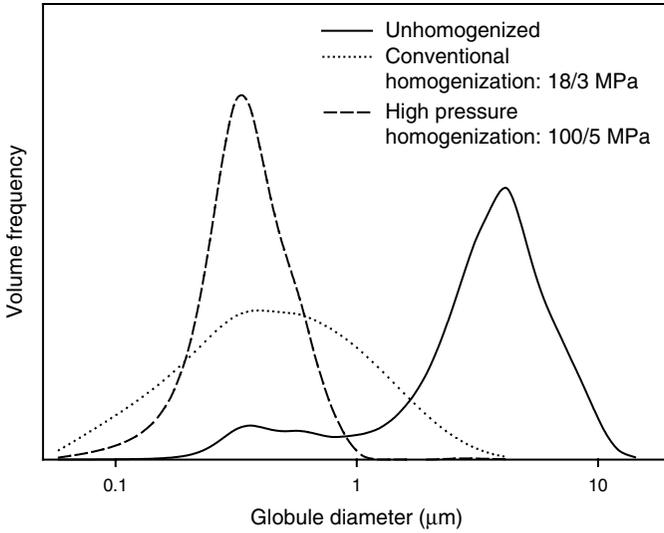
where  $\gamma$  is the interfacial tension and  $R$  is the radius of the droplet. The value for  $\gamma$  for native milk fat globules is very small, but increases as globules are deformed. To disrupt a droplet, it is necessary to apply an external force, which is considerably larger than  $P_L$  and the duration of application must be longer than the time required to deform and disrupt the droplet (McClements, 1999).

Globules can be deformed by the shearing action of liquid; if the viscous stress,  $\eta S$  (where  $\eta$  is the viscosity of milk serum and  $S$  is the velocity gradient), equals or exceeds  $P_L$ , the globule may be disrupted; this typically requires very high velocity gradients and, even then, the ratio of the viscosity of the milk fat to plasma protects all but the largest fat globules from shear-induced disruption, in cases of non-turbulent flow (Walstra, 1995). Disruption occurs more readily under turbulent flow conditions, depending on the amount of turbulent energy dissipated per unit time and per unit volume of liquid. Such conditions are encountered only very transiently (e.g., in the valve of homogenizers or at the top of a rapidly rotating stirrer blade).

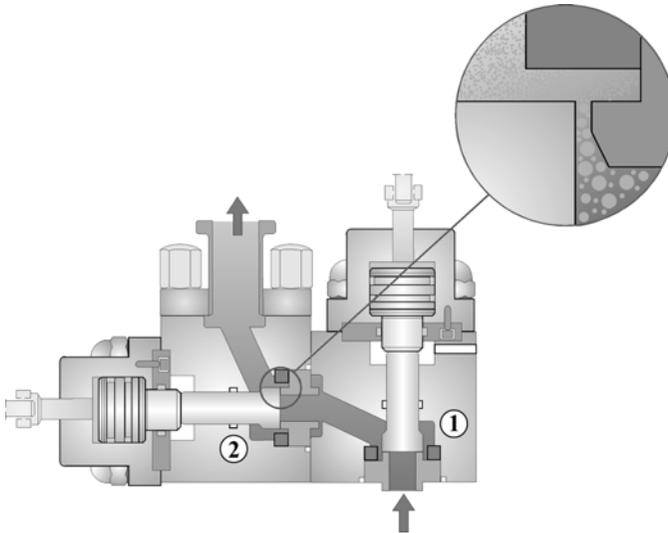
Homogenization is a process designed to reduce the size of the milk fat globules and thus retard separation of fat globules to such an extent that a cream layer does not form in homogenized milk products during their shelf-life. During homogenization, pre-warmed ( $\sim 40^\circ\text{C}$ ) milk (in which the fat is in a liquid state; homogenization is less effective when the fat is partially solid) is passed through a small orifice at a pressure of 10–20 MPa. Shearing, impact and distortion effects combine to stress the fat globules to such an extent that they split into a greater number of smaller globules (usually  $< 2 \mu\text{m}$  in diameter; Figure 5.4). The principle of operation of a valve homogenizer is shown in Figure 5.5. The extent of the reduction depends on a number of factors, including the geometry of the homogenizer valve used, the number of passes through the valve and, in particular, the homogenization pressure (Walstra, 1975). The relationship between  $d_{vs}$  and homogenization pressure ( $p_h$ ) is given by:

$$\log d_{vs} = k - 0.6 \log p_h$$

where the constant,  $k$ , varies between  $-2$  and  $-2.5$ , depending on the type of homogenizer and other processing conditions (Walstra, 1975). The principal mechanism responsible for globule disruption during homogenization is probably the occurrence of pressure fluctuations under turbulent flow conditions (Walstra, 1995). Creaming of fat globules in homogenized milk is considerably slower than the original globules, due to the reduction in fat globule size and the adsorption of milk proteins onto the fat globules, which increases their density and thereby decreases the rate of rising, as well as through inactivation of the homogenization-labile component involved in cold agglutination.



**Figure. 5.4.** Effect of two stage conventional (18/3 MPa) or high pressure (100/5 MPa) homogenization on the volume frequency distribution of fat globules bovine milk.



**Figure. 5.5.** Principle of operation of a typical two-stage homogenizer for liquid milk, indicating the first (1) and second stages (2). (Reproduced with permission from *Dairy Processing Handbook*, Tetra Pak Processing Systems AB, Lund, Sweden, 1995).

Michalski *et al.* (2002) studied the types of particles in homogenized milk and identified three classes:

- Disrupted globules covered mainly by caseins and some of the original MFGM material (the surface layer of fat globules in homogenized milk is discussed in Section 5.13).
- A population of very small native fat globules of around 100 nm in diameter that appeared unaffected by homogenization.
- Small newly formed lipid–protein complexes with a new casein-rich membrane, a type of particle also produced by ultrasonication or pumping. The presence of small fat globules that are apparently unaffected by homogenization may be explained by the fact that they are smaller than the “Kolmogorov scale” (Michalski *et al.*, 2002), which approximates the minimum size of particles that can be affected at a given homogenization pressure (i.e., 320, 240, 180 or 140 nm at 5, 10, 20 or 40 MPa, respectively) (Mulder and Walstra, 1974).

In recent years, novel homogenizing devices that reduce milk fat globule size considerably more than the traditional homogenizers by operating at a higher pressure (100–300 MPa; Figure 5.4), such as high pressure homogenizers (Hayes and Kelly, 2003a; Thiebaud *et al.*, 2003) and microfluidisers (McCrae, 1994; Strawbridge *et al.*, 1995; Hardham *et al.*, 2000) have been used. The operating principle of a high-pressure homogenizer is generally similar to that of a conventional two-stage mechanical homogenizer, but it operates at a significantly higher pressure. In a microfluidiser, forces are generated by impinging high-velocity fluid jets (Paquin, 1999). The forces exerted by high-pressure homogenization, including shear, cavitation, impacts, turbulence and frictional heating, can also kill bacteria, inactivate enzymes, denature whey proteins and alter several properties of milk (Hayes and Kelly, 2003a,b; Thiebaud *et al.*, 2003; Hayes *et al.*, 2005).

Ultrasonic treatment can also disrupt milk fat globules, probably through cavitation and other shear and shock effects (Villamiel, 1999). Wu *et al.* (2001) reported that high amplitude ultrasound homogenization of milk for yogurt manufacture achieved similar effects as conventional homogenization.

### 5.13. Milk Fat Globules in Homogenized Milk and Cream

The decrease in fat globule size on homogenization results in a significant increase in globule surface area. This new surface area is far too large to be covered and stabilized by the original amount of MFGM material; therefore, the surface becomes covered by casein micelles, or fragments

thereof, with some whey proteins also becoming attached if homogenization is combined with heat treatment (Darling and Butcher, 1978; Garcia-Risco *et al.*, 2002; Lee and Sherbon, 2002). The protein load per unit surface area increases as homogenization pressure is increased, but homogenization pressure has little effect on the composition of surface protein (Cano-Ruiz and Richter, 1997). The membrane in homogenized milk is thicker than that in fresh raw milk, and the proportion of casein in milk that becomes associated with the fat globules has been estimated to be ~6–8% (Fox, 2002). In extreme cases, a high protein load on the globules may lead to gravitational or centrifugal sedimentation instead of rise in evaporated milk (Fox *et al.*, 1960) or homogenized raw milk (Michalski *et al.*, 2002), respectively.

Fat globules in homogenized milk, due to their small size and the presence of a high level of casein on their surface, can, in effect, behave like casein micelles, which has significant implications for the heat-coagulation, acid-coagulation or rennet-coagulation properties of milk and hence for the properties of resulting products. These effects can be positive in the case of acidified milk products, such as yogurt, but they are generally undesirable in the case of cheese. Fat-casein complexes may accelerate the kinetics of coagulation, particularly heat coagulation; homogenization reduces the heat stability of whole milk (Sweetsur and Muir, 1983).

As discussed in Section 5.9, milk fat globules are naturally susceptible to partial coalescence or clumping. Freshly homogenized fat globules are particularly unstable and tend to coalesce into clusters or clumps (i.e., homogenization clusters); these clusters are formed as a result of the sharing of casein micelles between globules (even a single casein micelle can form a bridge between two neighboring globules), because the amount of surface-active material is insufficient to cover the newly formed interface (Ogden *et al.*, 1976; Darling and Butcher, 1978). The formation of homogenization clusters is usually prevented by use of a second, or even third, homogenization stage, usually at a lower pressure than the first stage (Kiesner *et al.*, 1997). The tendency to form homogenization clusters is enhanced by a high fat content, small fat globule size and a high surface protein load. Intense heat treatment before homogenization can also increase the tendency of globules to form clusters (Walstra, 1995).

The presence of homogenization clusters increases the viscosity of cream (Niar *et al.*, 2000), particularly during product storage; high-fat cream can acquire the consistency of a thick paste. This has implications for processes where only the cream is homogenized, followed by recombination with skim milk (e.g., liquid milk processing); if clusters form, products will cream readily. When fat is emulsified directly in skim milk, clusters are formed if the fat concentration exceeds 12% (w/w) (Oortwijn and Walstra, 1982b).

At a given homogenization pressure, average fat globule size decreases with increasing fat volume fraction up to a certain level, above which an increase is observed (Phipps, 1983), indicating the formation of homogenization clusters. In a conventional valve homogenizer, fat clustering in milk or cream occurred at a pressure  $> \sim 20$  MPa (McCrae, 1994; Noda and Yamamoto, 1996; McCrae and Lepoetre, 1996), whereas on high pressure homogenization, milk fat globule size decreased up to 250 MPa, but increased at 300 MPa, due to the formation of homogenization clusters (Thiebaud *et al.*, 2003). In a microfluidiser, some fat clustering occurred at 35 MPa, but none at 103 MPa (McCrae, 1994).

Homogenized milk is very susceptible to hydrolytic rancidity, as the protective function of the MFGM has been compromised (Iametti *et al.*, 1997); for this reason, homogenization should be combined with pasteurization, to inactivate lipases in milk. Several methods have been developed to evaluate damage to the MFGM (Iametti *et al.*, 1997; Evers, 2004). Homogenized milk is also more susceptible to the so-called “sunlight flavour” or “light-activated flavour” defect (Dunkley *et al.*, 1962). This flavor defect results from conversion of methionine to methional, catalyzed by riboflavin activated by photo-oxidation; however, the exact mechanism through which homogenization influences this process has thus far not been described. However, homogenized milk is less prone to copper-catalyzed lipid oxidation than unhomogenized milk (Tarassuk and Koops, 1960; Dunkley *et al.*, 1962), which is probably due to the fact that oxidation-susceptible phospholipids are more uniformly distributed throughout milk following homogenization, and are less likely to propagate lipid oxidation (Tarassuk and Koops, 1960).

#### **5.14. Milk Fat Globules in Recombined Milk**

Altered fat globule surface layers are of considerable importance in recombined milks. Typically, in the manufacture of recombined milk, skim milk powder, water and a source of milk fat (e.g., anhydrous milk fat; AMF) are mixed and homogenized to emulsify the fat and yield a stable product. Since AMF contains little, or no, MFGM material, the membrane surrounding the fat globules in recombined milk contains no original MFGM material; the nature of the new membrane is influenced strongly by adsorption conditions (e.g., composition of the continuous phase, agitation, temperature, heat treatment and fat:protein ratio). Both caseins and whey proteins are present in the membrane of recombined milk (Oortwijn *et al.*, 1977), but the proportion of whey proteins in the membrane is smaller than that in milk (Oortwijn and Walstra, 1982a; Sharma *et al.*, 1996a,b).

As the ratio of protein to fat in recombined milk is increased, the surface protein load on the fat globules increases; at higher ratios, little further effect is observed (Sharma *et al.*, 1996a). The protein load per unit of surface area of the fat globules in recombined milk is influenced markedly by the form of protein present in the continuous phase (i.e., it is markedly higher when casein micelles are present), than when only whey protein or sodium caseinate is present (Oortwijn and Walstra, 1979; Sharma and Singh, 1998, 1999). Furthermore, the surface protein load decreases with increasing temperature during emulsification (Oortwijn and Walstra, 1979; Sharma *et al.*, 1996a) and with increasing homogenization pressure (Sharma *et al.*, 1996a) and is increased by heat treatment of milk prior to emulsification (Oortwijn and Walstra, 1979; Sharma *et al.*, 1996a). Heat treatment prior to emulsification also increases the level of  $\beta$ -lactoglobulin in the membrane (Sharma *et al.*, 1996a).

The casein micelles on the globule surface in recombined milk often appear to be disrupted, which may be due either to homogenization or to the process of adsorption (Sharma *et al.*, 1996b). The extent of disruption of micelles in recombined milk is greater than that in freshly-homogenized milk (Sharma *et al.*, 1996b) and increases with the temperature of homogenization (40–70°C; Oortwijn and Walstra, 1982a). Disruption of casein micelles in recombined milk was not observed after fixation of casein micelles with glutaraldehyde or addition of the surfactant Tween 20 prior to homogenization (Oortwijn *et al.*, 1977); the latter effect is probably due to the preferential adsorption of Tween 20 over casein micelles on the micelle surface. Addition of surfactants before or after recombination also reduces the protein surface load (Oortwijn and Walstra, 1979). Destabilization of casein micelles by reducing the colloidal calcium phosphate content reduces the protein load on the fat globules in recombined milk and alters the proportions of individual caseins on the globule surface (Sharma *et al.*, 1996a). Sharma *et al.* (1996b) reported that it is far more difficult to remove  $\kappa$ -casein than  $\alpha_s$ -casein or  $\beta$ -casein from the fat globule surface in recombined milk, suggesting that part of the  $\kappa$ -casein is associated directly with the globule surface, which was confirmed by Su and Everett (2003).

Inclusion of certain emulsifiers prevents fat separation in UHT-processed recombined milk, with Tween 21 being most effective; refined monoglyceride actually enhanced creaming slightly, perhaps due to protein displacement from surface layers, thus reducing the effective density of the globules (Mayhill and Newstead, 1992). Addition of soy lecithin may reduce the stability of fat globules in recombined cream against coalescence (Melsen and Walstra, 1989).

Heating recombined milk products at 130°C at pH 6.7 leads to the formation of chains of fat globules and casein particles, linked *via* the latter.

Furthermore, at a  $\text{pH} \leq 6.7$ , the surface of the casein micelles on the fat globule surface develops appendages on heating, possibly whey protein aggregates, while those in milk of  $\text{pH} > 6.7$  remain free of whey proteins on heating (Singh *et al.*, 1996). Addition of AMF (without homogenization) or homogenization (in the absence of AMF) did not influence the heat coagulation time of the skim milk, but the heat stability of milk recombined from AMF and skim milk is considerably lower than that of the skim milk (Sharma and Singh, 1999). Furthermore, the heat stability of recombined milk decreased with decreasing fat globule size, which may be linked to a higher surface protein concentration and a lower proportion of  $\kappa$ -casein on smaller fat globules than on larger ones (Sharma and Singh, 1999). McCrae *et al.* (1994) reported that heat-induced interactions between whey proteins and casein adsorbed at the fat globule surface promote the heat-induced coagulation of recombined milk. The relatively low heat stability of recombined milk can be increased considerably by addition of soy lecithin, either pre- or post-homogenization (McCrae and Muir, 1992).

### 5.15. Free Fat

“Free fat” is a term used in the literature to denote a particular parameter that has been claimed to correlate with the degree of damage to, or stability of, fat globules; various definitions have been given for this ambiguous term (Evers, 2004), [e.g., “fat inside damaged globules” (Fink and Kessler, 1983), “fat that is enclosed insufficiently by an undamaged membrane” (Kessler and Fink, 1992) or “fat that has leaked out of globules” (Fink and Kessler, 1983)]. Other authors have defined free fat as a method-dependent parameter (e.g., the proportion of fat separated by centrifugation at  $60^\circ\text{C}$ ) (Te Whaiti and Fryer, 1975) or solvent-extractable fat (Deeth and Fitzgerald, 1978).

The question of whether free fat actually occurs in milk or cream is, in fact, controversial, with frequent suggestions that it is an artefact of the method used for its measurement (e.g., that organic solvents can damage the MFGM and extract some fat; Evers *et al.*, 2001). Walstra and co-workers (van Boekel and Walstra, 1989; 1995; Van Boekel and Folkerts, 1991; Walstra, 1995; Walstra *et al.*, 1999) suggested that there is more than sufficient protein in milk to cover any uncovered fat very rapidly (e.g., in  $\sim 10$  ms). The efficiency of methods to quantify the level of free fat was recently reviewed by Evers (2004), who, in agreement with Walstra (1995), concluded that these methods have poor repeatability due to their poor robustness (i.e., a very precise experimental control is required to obtain repeatable results). In some cases, the extraction method used may damage

the fat globules, thereby magnifying, or even generating, the extractable fat level (Evers *et al.*, 2001).

While free fat remains a controversial concept in liquid dairy products, it undoubtedly has an important role in whole milk powder (WMP); for certain applications (e.g., chocolate manufacture) high-free fat WMP is favored. Keogh *et al.* (2003) reported that the particle size in chocolate mixes after refining and the viscosity of the molten chocolate decreases as the free fat content of the WMP increased; such changes have significant implications for the mouthfeel and smoothness of chocolate. For more information on the role of fat in milk powder, see Chapter 13

### 5.16. Influence of Fat Globules on Rheological Properties of Milk and Cream

Rheological properties of emulsions are important in food science for various reasons. Some sensory attributes (e.g., creaminess, smoothness, thickness and flowability) of food emulsions are directly related to their rheological properties. Furthermore, the shelf-life of many food emulsions depends on rheological characteristics of the phases; for example, the rate of creaming of milk depends on the viscosity of milk plasma (McClements, 1999). The content of lactose or the whey proteins in milk influence the viscosity of milk only little; fat content has a major influence, although by far the greatest influence is that of the casein content (McCarthy, 2003). The influence of milk fat globules on the rheological properties of milk and cream, in particular the viscosity, will be discussed in this section.

If fat globules are present as separate particles, the fat content is  $\leq 40\%$  and the milk fat completely molten, milk and cream behave as Newtonian fluids at intermediate and high shear rates (Phipps, 1969; McCarthy, 2003), {i.e., its viscosity is not influenced by shear rate ( $\tau = \eta \times \gamma$ , where  $\tau$  is the shear stress [Pa],  $\eta$  is the viscosity [Pa s] and  $\gamma$  is the shear rate[1/s])}. For a Newtonian fluid, Eilers' equation (Eilers, 1941) is generally obeyed (Walstra, 1995):

$$\eta = \eta_0 \left[ 1 + \frac{1.25\phi}{1 - \phi/\phi_{\max}} \right]^2$$

where  $\eta$  is the viscosity of the product,  $\eta_0$  is the viscosity of the continuous phase,  $\phi$  is the volume fraction of spherical particles and  $\phi_{\max}$  is the hypothetical volume fraction when the particles are in the closest possible packing arrangement. Van Vliet and Walstra (1980) showed that if  $\phi$  is taken as

$\phi_{\text{fat}} + 0.16$  ( $\phi$  for casein micelles, lactose and whey proteins in skim milk  $\approx 0.16$ ),  $\eta_0 = 1.02\eta_{\text{water}}$  and  $\phi_{\text{max}} = 0.88$ , values calculated from Eilers equation are in good agreement with experimental data (Phipps, 1969).

At a temperature  $<40^\circ\text{C}$ , milk does not behave as a Newtonian fluid; the deviation from Newtonian flow becomes larger as the temperature decreases (Randhahn, 1973; Wayne and Shoemaker, 1988; Kristensen *et al.*, 1997). Viscosity of milk decreases with increasing shear rate at a temperature below  $40^\circ\text{C}$  (Randhahn, 1973), which Mulder and Walstra (1974) suggested may be due to disruption of clusters of milk fat globules, which were formed as a result of cold agglutination.

Rheological properties of milk and cream are influenced by various processes (e.g., heat treatment, cooling or homogenization). McClements (1999) reported that the main factors that determine the rheological properties of emulsions can be divided into five groups:

### 5.16.1. Volume Fraction of the Dispersed Phase

With an increase in dispersed-phase volume fraction, the viscosity of an emulsion increases. This increase in viscosity is linear at a low droplet concentration (McClements, 1999); the viscosity of an emulsion of milk fat globules in milk plasma increases linearly with fat content up to 30% (Bakshi and Smith, 1984; Kyazze and Starov, 2004), whereas the viscosity of low-fat milk ( $\leq 2.0\%$  fat) increases in a near linear fashion with fat content (Phillips *et al.*, 1995). However, above a certain volume fraction of the dispersed phase, the droplets in emulsions are packed so closely that flow is impaired, giving the emulsion a gel-like character (McClements, 1999). For instance, the viscosity of cream increases rapidly with increasing fat content when the fat content is  $>50\%$  (Prentice, 1968; Mulder & Walstra, 1974).

### 5.16.2. Rheology of the Component Phases

The viscosity of an emulsion is directly proportional to the viscosity of the continuous phase; any alteration in the rheological properties of the continuous phase results in a corresponding change in the rheology of the whole emulsion (McClements, 1999). The rheological properties of the dispersed phase (i.e., the milk fat globules in the case of milk and cream), have only a minor influence on the rheology of the emulsion (Walstra, 1996; McClements, 1999). This is illustrated well by the influence of temperature on milk viscosity. A decrease in temperature, particularly below ambient temperature, results in an increase in milk viscosity (Randhahn, 1973; Prentice, 1992; Kristensen *et al.*, 1997); even though considerable crystallization occurs of fat occurs in the globules on cooling, changing the rheological properties of the fat, increases in milk viscosity are almost completely due to

changes in the milk serum, primarily increases in the hydration of casein micelles (Prentice, 1992).

### 5.16.3 Droplet Size

The viscosity of a dilute emulsion is independent of the size of its droplets when long-range attractive and repulsive colloidal interactions between droplets are negligible and the thickness of the surface layer is small compared to the droplet diameter; however, when long-range colloidal interactions are present and/or the thickness of the surface layer is a significant proportion of particle diameter, particle size has a considerable effect on emulsion viscosity. The viscosity of an emulsion increases with increasing thickness of the surface layer, due to an increase in the effective volume fraction of the dispersed phase (Pal, 1996). Homogenization of milk leads to an increase in milk viscosity (Whitnah *et al.*, 1956; Randhahn, 1973), which Prentice (1992) suggested is due to the adsorption of casein particles on the fat globule surface, thereby increasing the effective volume fraction of the dispersed phase. The formation of homogenization clusters also leads to increases in viscosity of the product, as discussed in Section 5.13.

### 5.16.4. Colloidal Interactions

Colloidal interactions between emulsion droplets play a primary role in determining emulsion rheology. If attractions predominate over repulsive forces, flocculation can occur, which leads to an increase in the effective volume fraction of the dispersed phase and thus increases viscosity (McClements, 1999). Clustering of milk fat globules due to cold agglutination increases the effective volume fraction of the milk fat globules, thereby increasing viscosity (Prentice, 1992).

### 5.16.5. Particle Charge

The charge on an emulsion droplet can influence the rheological properties of the emulsion. Firstly, the charge determines whether droplets tend to aggregate (see Section 5.6). Furthermore, droplet charge can also influence the rheological properties of an emulsion through the primary electroviscous effect (Pal, 1996); movement of a charged droplet through a fluid results in distortion of the surrounding cloud of counter-ions, which causes an attraction between the charged droplet and the cloud of counterions that lags slightly behind it. This attraction opposes the movement of droplets and thus increases the viscosity of the emulsion because more energy is needed to cause droplets to move (Pal, 1996; McClements, 1999). This mechanism may

be involved in the increase in viscosity observed on homogenization of milk (Whitnah *et al.*, 1956; Randhahn, 1973). The fat globules in homogenized milk have a higher net negative surface charge than those in unhomogenized milk (Dagleish, 1984; Michalski *et al.*, 2001b), which may, through the primary electroviscous effect, result in increased milk viscosity, although this has to be confirmed with experimental data.

Overall, it is apparent that, although fat globules are not the predominant milk constituent affecting the rheological properties of milk and cream, they still exhibit a considerable influence.

## 5.17. Conclusions

Whole milk or cream can be regarded as an emulsion of milk fat globules in milk plasma. The physico-chemical properties of the milk fat globules affect many properties of liquid dairy products such as milk and cream, and as such should always be considered when studying the stability of liquid dairy products. The physicochemical properties of the milk fat globules can be influenced through a wide variety of processes, as described in this chapter and, once chosen and controlled carefully, these processes can be efficiently used to give products desired characteristics (e.g., in terms of storage stability or rheological properties). Although much is known concerning physico-chemical properties of the milk fat globules, and instability of dairy emulsions can be controlled well with the current state of knowledge, gathering further information concerning the physical chemistry of milk fat globules, and the underlying fundamental problems, remains crucial. Pursuit of fundamental knowledge often leads to good results, sometimes in unexpected ways. Thus, it is important to continue to enhance our understanding of areas such as those described in this chapter.

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# Composition, Applications, Fractionation, Technological and Nutritional Significance of Milk Fat Globule Membrane Material

R.E. Ward, J.B. German and M. Corredig

## 6.1. Introduction

Among the components of mammalian milks, the milk fat globule membrane (MFGM) is the least close to being fully understood. As a result of the synthesis and secretion process, the fat globules in milk are composed of a non-polar lipid core, surrounded by a layer of phospholipids and proteins. This structure is then bounded by a membrane bilayer that is, in turn, derived from the apical surface of the mammary epithelial cell. Substantial biochemical investigations have been conducted to elucidate the details involved in the synthesis, transport and secretion of milk fat globules, yet there has been little discussion on the uniqueness of this system, and the evolutionary forces that have led to its development. On one hand, the complex structure of the globules may have arisen as a result of the physiological constraints on the secretion process, and would therefore not be expected to contribute a significant benefit to the health of the offspring. It should be recognized, however, that the simple process of lipoprotein secretion, which does not require the addition of extra cell bilayer surface, had evolved well before the appearance of mammals and would presumably have been a simple and easy alternative for fat secretion. Thus, it is also possible that there is a nutritional or physiological benefit that may be conferred to

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

the consumer of milk by the structure of the fat globules, which would have led to the Darwinian selection of this process during mammalian evolution.

According to evolutionary theory, individuals of a species tend to be selected over time as a function of survival and reproductive success, and hence pass on their genetic material. Parturition is a tenuous time in the mammalian life cycle, as the constant threat of microbial pathogens is compounded by developmental delays in mucosal and systemic immunity (Goldman, 2002). However, the biological role of milk is to provide a support system that promotes metabolic regulation, assists in rapid physical development and complements the functionally immature immune system. Consequently, infants who consume breast milk experience phenotypic benefits, which we cannot explain with our current understanding of nutrition, food composition and structure. Although alternate formulae match the macronutrient, vitamin and mineral composition of breast milk, it must be the composite, non-essential structures in milk, not present in formulae, which are responsible for this extent of differential benefit. The milk fat globule membrane is composed of an interesting mixture of proteins, lipids and carbohydrates. With its similarity to epithelial cell surfaces, it may well have functions and activities above the simple delivery of the nutrients it contains.

In food and nutrition research, there is growing awareness of dietary components, which, while not essential, nonetheless provide tangible benefits to health when consumed. Many of the components of the MFGM have been associated positively with health, whether or not the mechanism of action is understood. Importantly, the identification and subsequent characterization of the constituents responsible for these effects will not be as straightforward as was the scientific research on the essential nutrients. If a nutrient is essential, removing it from the diet will always result in specific, reproducible insults to health. Yet, food science and nutrition research has not yet been as successful in developing research protocols to probe the importance and function of conditionally essential nutrients. As milk contributes to the survival and fitness of mammalian young, it is not simply a participant in evolution, but rather a driver, with simultaneous selection for efficacy and efficiency. Therefore, it is hard to imagine that there is anything in milk, including the MFGM, which is not interesting from a nutritional perspective.

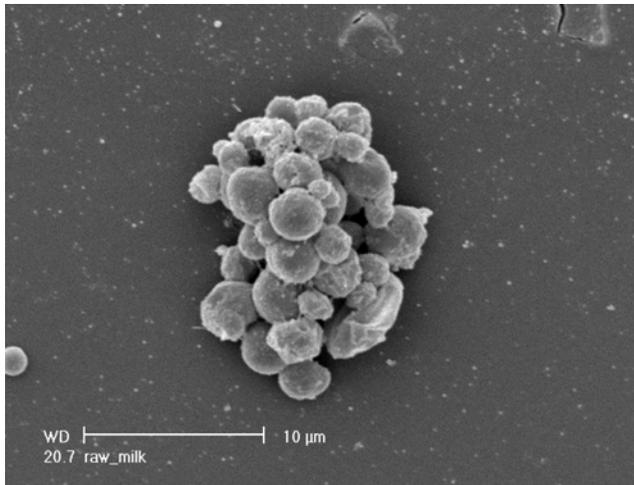
## **6.2. Nutritional and Physiological Significance of the Milk Fat Globule Membrane**

Most of the literature on milk fat globules has been generated using bovine, goat or human milk, and thus it is possible that alternate lipids may be present in other species. However, to date this has not been reported. The

lipids in milk are present as colloidal particles, due to their thermodynamic incompatibility with the aqueous serum, and the assembly of these insoluble components into stable multi-molecular units is the result of a unique secretory process. Milk fat globules are structurally complex, as a result of their synthesis and secretion, and it is possible to see many levels of nutritional value in the various stages of their architecture. The fat globule is primarily a core of triacylglycerides synthesized within the endoplasmic reticulum of the mammary epithelial cell. This core of triglycerides is bounded by a monolayer of polar lipids, such as phospholipids, derived from the endoplasmic reticulum membrane of the cell. This layer is also characterized by a dense proteinaceous coat located on the outside of the triglyceride core. The entire globule, however, is surrounded by a bilayer plasma membrane, which enrobes the globule as it exits the epithelial cell (Keenan and Mather, 2002). The MFGM is composed of the lipids and proteins of the epithelial cell plasma membrane, including significant quantities of cholesterol, phosphatidylcholine and sphingomyelin. Additional components unique to the external surface of the native fat globule include glycolipids, gangliosides and significant quantities of membrane glycoproteins and mucins. The membrane helps to stabilize the fat globules as an emulsion in the aqueous environment of milk. The average diameter of the milk fat globules ranges from less than 1  $\mu\text{m}$  to 10  $\mu\text{m}$  (Jensen, 2002), with three main size populations (Keenan and Patton, 1995). The small globule distribution is centered at less than 1  $\mu\text{m}$  in diameter, the intermediate globule size distribution at roughly 3–5  $\mu\text{m}$ , and large globule distribution at about 8–10  $\mu\text{m}$ . Figure 6.1 is a scanning electron microscope image of milk fat globules from bovine milk. Most of the globules visible in the micrograph fall in the intermediate size range.

The colloidal properties of fat globules give rise to interesting compositional features. Numerically, most globules in milk (70–90%) are less than 1  $\mu\text{m}$  in diameter and yet account for less than 5% of the total milk lipid, whereas those of intermediate size account for 10–30% of the globules, yet 90% of the total lipid. The proportion of polar lipid (phospholipids) surrounding the core of neutral lipids (triglycerides, cholesterol esters) increases as the globule size decreases but in a globule of 1  $\mu\text{m}$  diameter, the MFGM accounts for only 3% of the volume. In a 0.5  $\mu\text{m}$  diameter globule, the membrane makes up 6% of the globule volume. Although the membrane makes up between 1 and 5% of the lipid fraction, the surface area of the fat globules in 1 ml of mature human milk is estimated to be 500  $\text{cm}^2$  (Ruegg and Blanc, 1981). In colostrum, in which the fat occurs almost exclusively as very small globules, the proportion of membrane lipids is even higher.

To date, little attention has been given to the native structural properties of milk lipids. In particular, little is known about how these structural



**Figure 6.1.** Scanning electron micrograph of a cluster of fat globules from fresh, unhomogenized whole milk. Sample was fixed with glutaraldehyde and post-fixed with  $\text{OsO}_4$ .

features relate to the nutritional and functional properties of particular components and the overall properties of milk as an intact delivery system. The fact that these structures vary widely, but consistently, among all mammalian species and at different periods of lactation implies that structure is of functional value. However, the techniques necessary to describe the structures of lipids are not fully developed. As these techniques become available, research must address how variations in structure influence biological and nutritional properties.

### 6.2.1. Biological Significance of Native Globules

The structure and size distribution of milk fat globules affects the presentation of the membrane surface, and the rate of lipolysis. In addition, there are discrete compartments within the globule, which may have functional consequences. It has long been supposed that the presence of a vast MFGM surface area in milk can serve as a decoy for intestinal pathogens that seek to adhere to epithelial cells. Certain constituents have been associated with binding of pathogens, such as the mucins, lactadherin and gangliosides. Binding to host epithelial tissues is a prerequisite for infection by some pathogens, and for many, the cognate ligands for their bacterial adhesion are complex glycans. Indeed, as the surface of milk fat globules is derived from the apical membrane of epithelial cells, it presents a glycan-rich glycocalyx in milk, similar to the host epithelial cells. In colostrum, the

majority of the globules are small, and this increases the surface area to lipid ratio. Not surprisingly, colostrum is also the richest source of other substances involved in protection against pathogens, such as the immunoglobulins and lysozyme. Although the non-covalent interactions between proteins and carbohydrates are of low affinity and thus have equilibrium constants in the millimolar range, multiple interactions of receptors and ligands increase binding avidity, and make the attraction stronger.

A second functional consequence of the structure of milk fat globules is its effect on lipolysis. Of the three lipases (gastric, pancreatic, and bile salt-stimulated) that act on triglycerides in human infants, only gastric lipase is able to penetrate the lipid bilayer of the MFGM to initiate digestion (Bernback *et al.*, 1989). Without the prior action of this enzyme in an *in vitro* assay, the ability of the two other lipases to release fatty acids from milk fat globules is greatly reduced. Furthermore, as evidenced by studies on rats, this enzyme is more active on triglycerides containing short-chain fatty acids, which are antimicrobial, and which are absorbed directly in the stomach (Jensen, 1989). It has not been reported whether or not the human gastric lipase is more active on triglycerides containing short-chain fatty acids. Putting these data together, it is tempting to speculate that the native structure of the milk fat globule along with the specificity of gastric lipase leads to selective hydrolysis of short-chain fatty acids in the stomach where they provide protection against pathogens, and also provide a ready source of energy. The result of this enzymatic activity would then leave the fat globules more susceptible to hydrolysis by pancreatic and bile-salt stimulated lipases in the small intestine.

In some milk fat globules, small aqueous compartments are located beneath the membrane bilayer, which have been termed cytoplasmic crescents (Huston and Patton, 1990). Whether or not this cytoplasmic inclusion provides some benefit is unknown. Yet, as this aqueous compartment is protected from the bulk serum phase by the MFGM, constituents located therein are presumably afforded some protection, at least initially, from gastric hydrolysis. Huston and Patton (1990) found crescents in all samples of milk they examined, and they were more prevalent in human (7.3% of globules), than in bovine (1% of globules) milk. Furthermore, there was considerable individual and diurnal variation. The structure of a cytoplasmic inclusion, surrounded by an intact plasma membrane on one side and a fat globule surface on the other, may allow certain labile constituents to be protected until they reach their proper site of bioactivity. At this point it is not known whether the crescents have a purpose or are simply the result of inefficiencies in the secretion process. As it is possible to isolate milk preparations enriched in cytoplasmic crescents, there is an opportunity to determine the nature of the materials found within. This unusual biocompartment may prove to be a model of food structure for biodelivery.

### 6.2.2. MFGM Consumption Studies: Physiological and Nutritional Effects

Considering the large quantity of bovine MFGM that is produced industrially each year as a byproduct in butter churning, and is available as a food ingredient with a unique polar lipid and membrane protein profile, few studies have been conducted to assess the physiological and nutritional effects of its consumption. Its presence in milk suggests beneficial bioactivities, and this perspective is reinforced by analysis of the effects on health of its individual components. However, feeding studies are still necessary to provide scientific evidence in support of synergisms, or perhaps antagonisms, and resolve whether putative benefits can be detected on the system under investigation.

Not surprisingly, most of the studies performed on the health aspects of consuming the MFGM during the past three decades have investigated solely its effect on serum cholesterol. Several studies have indicated that the consumption of MFGM lowers serum cholesterol, but other studies failed to reproduce this effect. After noting low serum cholesterol in the East African Masai, a group with a large daily milk intake, Mann (1977) reported that the consumption of four liters of whole milk per day lowered the level of serum cholesterol, and reduced the incorporation of radiolabelled acetate into cholesterol. Howard and Marks (1977) fed individuals various milk fractions in an attempt to identify the causal factor. After a two-week study, they noticed that butterfat (80 g/day) significantly raised serum cholesterol, whereas an equal amount of fat as cream did not. Furthermore, spray-dried buttermilk significantly reduced the level of serum cholesterol. The authors concluded that the effect on serum cholesterol may involve the MFGM. To test this hypothesis explicitly, Antila *et al.* (1980) fed volunteers either cultured buttermilk or cultured skim milk, and found that while both lowered serum cholesterol, the former was more effective. The cultured buttermilk in this study was derived from the aqueous byproduct of butter production, is rich in MFGM, and should not be confused with the commercially available cultured buttermilk, which is produced from skim milk.

The diets used in these initial studies on the effect of consuming buttermilk on serum cholesterol were not standardized, and involved a small number of individuals. Hussi *et al.* (1981) fed a large group of healthy volunteers with either 2.7 L/day of skim milk or 2 L/day of buttermilk or a control diet for three weeks. All diets were standardized for macronutrient and energy level, and all volunteers consumed the control diet for 3 weeks prior to the study. No significant differences were found in the serum lipid or lipoprotein profiles between the control and test groups.

The ability of MFGM to inhibit intestinal  $\beta$ -glucuronidase activity was measured by Ito *et al.* (1993). This enzyme is a product of colonic

enterobacteria, and when present in the colon has the ability to activate carcinogenic precursors to carcinogens (Simon and Gorbach, 1984). Since this activity is inhibited by sialoglycoproteins from porcine salivary glands (Sakamoto, 1974), the possibility that sialoglycoproteins in bovine milk might provide this benefit was investigated by Ito *et al.* (1993) in *in vitro* enzymatic assays and, in *in vivo* feeding studies with mice. In the enzyme assay, 0.2%, w/v, purified MFGM inhibited  $\beta$ -glucuronidase activity on phenolphthalein- $\beta$ -D-glucuronide by 90%, while  $\kappa$ -casein at the same concentration inhibited the reaction by 35%. To determine whether this activity would survive gastrointestinal transit, the authors fed mice diets supplemented with 5, 10 or 20% MFGM for five days, and measured  $\beta$ -glucuronidase activity in faeces. The diets containing 5 or 10% MFGM caused 15–20% inhibition of faecal  $\beta$ -glucuronidase activity, whereas when 20% MFGM was added to the standard diet, the level of inhibition was 50%.

### 6.3. Composition and Bioactivity of Individual Components

In addition to being an essential structural component (emulsifies) of milk fat globules, the MFGM is composed of many molecules that have been associated both individually and collectively with beneficial nutritional bioactivities. Although very few of the constituents of MFGM preparations are essential in our diet, many components are increasingly being documented as providing specific nutritional benefits. A discussion of the current nutritional understanding of the major constituents of the MFGM follows.

#### 6.3.1. Phospholipids

The MFGM is a rich source of phospholipids. Dietary phospholipids have antioxidative activity (Saito and Ishihara, 1977), as well as antimicrobial and antiviral properties (van Hooijdonk *et al.*, 2000). Additionally, there is evidence that the consumption of phospholipids can protect against gastric ulceration (Kivinen *et al.*, 1992). In a rat model for the ulcerative action of HCl administered to the stomach lumen, raw rat or bovine milk, and also pasteurized-homogenized bovine milk provided protection. However, this effect was not found when phospholipids were removed from the milk (Dial and Lichtenberger, 1984). In the duodenum, dietary phospholipids are converted to their lyso-forms by phospholipases and, to a lesser extent, by pancreatic lipase. Lysophospholipids are strong surfactants, and can cause lysis of Gram-positive bacteria. Sprong *et al.* (1999) tested the effects of phosphatidylcholine, phosphatidylethanolamine and their lyso-forms on *Listeria monocytogenes* in cultured cells *in vitro* and in rats fed diets based on lactase-treated sweet buttermilk powder. *In vitro*, lysophosphatidylcholine

inhibited the growth of *L. monocytogenes*, and in rats orally infected, the number of luminal and mucosal bacteria was significantly reduced by a buttermilk diet compared with a skim milk diet. These investigators concluded that buttermilk phospholipids might improve the resistance of the host to infection by *L. monocytogenes* by enhancing the gastrointestinal killing of this pathogen.

Milk fat contains small amounts of ether lipids, which include alkyl-diglycerols and alkylglycerophospholipids (reviewed by Parodi, 1996). In these molecules, the *sn*-1 position of glycerol has an ether-linked acyl chain, compared to an ester-linked chain in phospholipids. While the non-polar lipids of milk contain 0.01% by weight of these molecules, the phospholipid fractions contains up to 0.16% by weight (Hallgren *et al.*, 1974), and presumably they partition with the MFGM in the churning of butter. Human milk contains 10 times more ether lipids than bovine milk (Hallgren and Larsson, 1962). In the intestinal lumen of rodents and humans, dietary ether lipids are converted to *sn*-1-monoalkylglycerols and absorbed, with the ether linkage intact. They are then transported to the liver and used to synthesize membrane alkylglycerolipids and plasmalogens (Blank *et al.*, 1991; Das *et al.*, 1992). The biological effect of ether lipids is believed to be a result of their influence on the properties of membranes. They have been shown to have anticancer effects, by preventing growth and metastasis, and preventing induction of differentiation of tumors (Berdel, 1991; Diomedea *et al.*, 1993).

### 6.3.2. Ceramide Sphingolipids and Glycosphingolipids

Sphingolipids are not essential nutrients but are increasingly being recognized as important in nutrition, as was reviewed by Vesper *et al.* (1999). In mammalian tissues and milk, the sphingolipids include ceramides, sphingomyelins, cerebrosides, gangliosides and sulfatides.

Both sphingolipids and gangliosides are present in human and bovine milk and are enriched in products such as cream and cheese. The dominant phospholipid in milk is sphingomyelin, and it is reported to represent about one-third of total bovine milk phospholipids (Pfeuffer and Schrezenmeir, 2001), and 38% of total human milk phospholipids (Motouri *et al.*, 2003). Unlike phospholipids, which are built on a phosphoglycerol backbone, sphingolipids are based on sphingosine, an amino alcohol with a long unsaturated hydrocarbon chain. Whereas in phospholipids both acyl chains are linked to the phosphoglycerol backbone by ester bonds, sphingolipids have one acyl chain linked *via* an amide bond to sphingosine. This core of sphingolipids and gangliosides, *N*-acylsphingosine, is also known as a ceramide. Sphingolipids also contain a polar group, such as phosphocholine; gangliosides are further derivatized on the polar head groups with addition

of neutral and acidic sugars. Sphingolipids are among the most structurally diverse categories of lipids in nature.

The profile and concentration of gangliosides in milk vary across mammalian species. GD3 and GM3 are the predominant gangliosides in bovine milk, whereas in human colostrum, GD3 predominates and in mature human milk, GM3 predominates (Rueda *et al.*, 1998). GM1 has been shown to prevent diarrhea caused by *Escherichia coli* and *Vibrio cholerae* enterotoxins. Furthermore, a ganglioside-supplemented infant formula modifies the intestinal ecology of pre-term infants, increasing the numbers of *Bifidobacteria* and lowering that of *E. coli*. The proposed mechanism of action is that soluble membranes, like that on milk fat globules, serve as false intestinal receptors for some strains of pathogenic bacteria. GD3 and other gangliosides are involved in mechanisms of lymphocyte activation and differentiation, and thus milk gangliosides might function in the development of intestinal immunity. The levels of neutral glycolipids and other glycosphingolipids in bovine milk have been tabulated, with references, by Jensen (2002).

The *per caput* sphingolipid consumption in the U.S. is estimated to be 150–180 mmol (~115–140 g) per year, or 0.3–0.4 g/day (Vesper *et al.*, 1999). Though there is no nutritional requirement for sphingolipids (Vesper *et al.*, 1999; Berra *et al.*, 2002), studies in a rat model indicate significant benefits at particular life stages, for example in promoting gut maturation in the suckling infant. Suckling rats fed 0.5% sphingomyelin had a significantly lower level of intestinal lactase, vacuolated cells in intestinal villi were restricted to the tip of villi, and the Auerbach nerve plexus area of the ileum was significantly greater than in the control group. These results suggest that sphingomyelin plays an important role in neonatal gut maturation during the suckling period. In additional studies (Oshida *et al.*, 2003), suckling rat pups were injected daily with an inhibitor of sphingolipid biosynthesis from 8 days after birth (2 days before the onset of myelination) to 17 days after birth. The experimental group was then fed supplemental sphingomyelin until 28 days of life. Lipid analysis and morphometric analyses of the optic nerve showed that dietary sphingomyelin contributed to myelination of the developing rat central nervous system.

Reports indicate that the digestion and delivery of exogenous sphingomyelin to the intestinal cells and the interaction of these dietary components with endogenous sphingomyelin in the intestinal mucosa are relevant to optimal cell regulation and preventing such defects as colon cancer (Duan, 1998). There is experimental evidence that the consumption of sphingolipids inhibits the early stages of colon carcinogenesis, as determined by the appearance of aberrant crypt foci in mice (Dillehay *et al.*, 1994; Schmelz *et al.*, 1996, 1997, 2000). Dietary sphingolipids also reduce serum LDL-cholesterol

and elevate HDL (Imaizumi *et al.*, 1992; Kobayashi *et al.*, 1997). These findings have been interpreted to indicate that sphingolipids are “functional” components of foods.

The digestion products of sphingolipids (ceramides and sphingoid bases) are highly bioactive compounds that regulate cell growth, differentiation and apoptosis (programmed cell death), all of which are processes that are lost in cancer (Merrill *et al.*, 2001). Sphingolipids are involved in functions that range from structural protection to signal transduction and protein sorting, and participate in lipid raft assembly (Slimane and Hoekstra, 2002). Cholesterol-sphingolipid microdomains (lipid rafts) are part of the machinery that ensures the correct intracellular trafficking of proteins and lipids (Ikonen, 2001).

Smith and Merrill (2002) presented a mini-review prologue for a series covering the current understanding of sphingolipid biosynthesis, intracellular transport and turnover. In this mini-series, Merrill (2002) discussed the fact that *in vivo* biosynthesis of sphingolipids is probably required although sphingolipids are present in most foods, including the MFGM. The *de novo* pathway must be controlled because so many of the intermediates are highly bioactive, especially ceramides, which are the immediate precursors of sphingomyelins and glycosphingolipids and are one of the important mediators in signalling cascades of apoptosis, proliferation and stress responses (Hannun and Obeid, 2002; Spiegel and Milstien, 2002). In the same mini-series, van Meer and Lisman (2002) reviewed the role of sphingolipids in the structure of membrane rafts, sphingolipid biosynthesis, and translocators important in directing sphingolipid distribution in cells.

Recently, milk sphingomyelins were reported to interact significantly with the physical state of cholesterol, which correlated positively with reduced uptake and esterification of cholesterol by Caco-2 cells; they also significantly reduced cholesterol absorption in mice, even at 0.1% of the diet (Eckhardt *et al.*, 2002). An earlier study showed the regulation of cholesterol absorption by the content of sphingomyelin in intestinal cell membranes (Chen *et al.*, 1992).

Sphingolipids have also been implicated as mediators of bone health. As a testament to their bioactive potential, two recent patents (Takada *et al.*, 2001a,b) describe their use as drugs. One drug containing ceramides, sphingomyelins, sphingoglycolipids, and gangliosides is for the treatment of periodontal diseases. Application of a solution containing gangliosides prepared from milk to the teeth of hamsters with experimentally-induced periodontal disease significantly suppressed the decrease in alveolar bone.

The second patent defines drugs for the prevention and treatment of osteoporosis, fracture, lumbago, and rheumatic arthritis. These drugs contain compounds such as ceramides, sphingomyelins, sphingoglycolipids and

gangliosides, and optionally calcium, vitamin D and/or vitamin E. In ovariectomized osteoporotic rats fed diets containing gangliosides prepared from milk, milk calcium and vitamin D, the decrease in the amount of bone minerals was suppressed.

In a commercial response to the potential value of sphingolipids as functional food components, a patented process for preparing milk products enriched in both phospholipids and sphingolipids was developed (Dewettinck and Boone, 2002). These products are obtained by ultrafiltration of byproducts from the direct processing of milk or from the further processing of directly acquired byproducts. The ultrafiltration membrane used had a cut-off value ranging from 5,000 to 20,000 Da.

### 6.3.3. Proteins

Although the proteins in the MFGM represent only about 1% of the total milk protein, they are unique in their functionality. Identification of the component proteins is based largely on electrophoretic mobility, more specifically on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The composition, structure and function of the proteins and glycoproteins in bovine MFGM has been reviewed extensively (McPherson and Kitchen, 1983; Kanno, 1990; Danthine *et al.*, 2000; Mather, 2000; see Chapter 4). Mather (2000) referred to the complex banding pattern of MFGM proteins separated on two dimensional (2D) gels as evidence of the complexity of the membrane proteome. Many of the more quantitatively abundant proteins have been characterized, and Mather (2000) provided detailed information on eight proteins, from the primary sequence to post-translational modifications. The major proteins in bovine MFGM are: mucin MUC1, xanthine dehydrogenase/oxidase, PAS III, CD36, butyrophilin, a group of glycosylated proteins called PAS 6/7, and adipophilin (see also Chapter 4).

In addition to the major proteins, many bioactive constituents work at low concentrations, and thus it is of interest to catalogue and functionally annotate all of the proteins present. To that end, a few proteomic studies have been conducted on human milk fat globules using 2D-PAGE gels coupled with subsequent identification using mass spectrometry (Quaranta *et al.*, 2001; Charlwood *et al.*, 2002; Fortunato *et al.*, 2003). Global proteomic investigations are also helping to unravel the assembly process of milk fat globules. Wu *et al.* (2000) separated murine proteins from cytoplasmic lipid droplets on 2D-PAGE gels, digested them with trypsin, and then identified the resulting peptide fragments using tandem mass spectrometry. By comparing the gel pattern of mammary cytoplasmic lipid droplets to that of the milk fat globules and liver cytoplasmic lipid droplets, they were able to identify proteins that are likely mediators of the cross-cell lipid traffic.

#### 6.3.4. Butyrophilin

Butyrophilin, the most abundant protein in bovine MFGM, has a molecular weight of 66 000 Da. Although the function of butyrophilin is still subject of active debate, the protein is a member of a large family of immunoglobulins related to activity of the immune system. Most butyrophilin is found associated with the membrane and seems to be an integral protein. Sequence homology studies have indicated that domains of butyrophilin are highly conserved among species and may have a universal function in protein-protein interactions. It was hypothesized that butyrophilin and Xanthine oxidoreductase play an important role in the formation of a supermolecular complex (also with adipophilin), which may be an essential step in the assembly of the MFGM and the secretion of the fat globules (Mather and Kennan, 1998; see Chapter 4). Disulfide bonds play a role in stabilizing its association with the membrane. The levels of butyrophilin and Xanthine oxidoreductase are highest in early lactation and then decrease as lactation progresses to its midpoint. The molar ratio is reported to be between 4:1 and 3:1 (Mondy and Keenan, 1993; Ye *et al.*, 2002). Direct evidence of a thiol-dependent complex between Xanthine oxidoreductase, butyrophilin and adipophilin has been shown in support of the hypothesis that membrane association of the most abundant MFGM proteins is crucial for the secretion process of the mammary epithelial cells (McManaman *et al.*, 2002).

Although the function of butyrophilin is under debate, epidemiological associations have been interpreted to suggest that it may be involved in the etiology of the autoimmune disorders, Multiple Sclerosis (MS) and autism. MS is an inflammatory autoimmune disease of the central nervous system that results in demyelination of neurons due to a disruption of immunological self-tolerance (Mana *et al.*, 2004). The mechanistic cause of MS remains completely unknown and a variety of hypotheses have been proposed and tested. One hypothesis is that immunogenic determinants of important (self) proteins can be mimicked by food-based molecules, and this is the proposed mechanism for the involvement of butyrophilin. Indeed, there seem to be roles for both genetic predisposition as well as environmental factors in triggering the onset of the disease, making it difficult to discover a single casual agent or mechanism behind the disease. Epidemiological studies seem to suggest that the consumption of dairy products may be one of many environmental factors associated with higher rates of MS in susceptible individuals (Lauer, 1997). Whereas a biological effect does seem plausible according to the data from some of the studies, many confounding factors are present. Further studies are needed using randomized trials to substantiate the relationship between MFGM and the disease (Lauer, 1997).

Butyrophilin contains an Ig-like domain that is common to an extended family of B7-like proteins. Another member of this family is the myelin oligodendrocyte glycoprotein (MOG), which is a central nervous system protein thought to be an important target of the autoimmune response in MS. The implication of butyrophilin in the etiology of MS comes as a result of its greater than 50% peptide sequence identity to MOG. Furthermore, in an animal model for the disease, experimental autoimmune encephalomyelitis (EAE), MOG is a major target of the autoimmune response (Stefflerl *et al.*, 2000). In Dark Agouti rats, which are genetically susceptible to EAE, the authors have shown that the CD4<sup>+</sup> T cell response to intravenously injected MOG is mutually cross reactive with the IgV-like extracellular domain of native butyrophilin, and *vice versa*. Such cross reactivity of endogenous and exogenous epitopes is known as “molecular mimicry,” and has been suggested to be the cause of other autoimmune disorders. Stefflerl *et al.* (2000) also found that transmucosal exposure (*via* intranasal administration) to both the MOG and the butyrophilin antigens modulated the severity of MOG-induced EAE. This finding indicates that dietary butyrophilin may actually promote oral tolerance to the antigen, and through cross-reactivity may suppress autoimmune MOG-reactive T-cells. Speculating about a plausible role for butyrophilin in inducing MS, the authors point out that oral tolerance is poorly developed at birth, and exposure to dietary antigens at this time could activate the immune system, rather than provide tolerance.

Mana *et al.* (2004) have shown that the link between molecular mimicry, environmental antigens and autoimmunity does not necessitate pathogenesis, building on the findings that transmucosal exposure to antigens can modulate the immune response. Working with C57BL/6 mice, a strain genetically susceptible to EAE, they found that treatment with butyrophilin before immunization with MOG can prevent pathogenesis, and treatment after MOG immunization can suppress the disease symptoms. The effect seemed to lie in the reduction of immune cell proliferation, and the reduction of Th1 related cytokines, IFN- $\gamma$ , IL-2, IL-12 and GM-CSF, with an up-regulation of IL-10. One important finding of this study is that the existence of self-reactive antibodies does not lead to autoimmunity.

The cross-reactivity of an autoimmune antibody to butyrophilin has also been described in autism, a developmental disorder whose etiology includes genetic, environmental, neurological and immunological components. Using an enzyme-linked immunosorbent assay, Vojdani *et al.* (2002) demonstrated higher levels of antibodies against nine different neuron-specific antigens, two microbial antigens and butyrophilin in the serum of autistic children versus non-autistic controls. While this study confirms the observation in the MS studies that antibodies raised to MOG will cross-react

with butyrophilin, these findings do not indicate that it was butyrophilin, and not MOG, to which the immune response was initially raised.

In both the MS and autism cases, more information is needed on the digestion and survival of milk protein-derived peptides in the gut, their interaction with the gut-associated lymphoid tissue, the microflora, and their role in the disease etiology. Most critically, the basic mechanistic understanding of immune development remains poor. The suggestion by some scientists that dietary butyrophilin may play a role in promoting MS and autism, and the inability of other scientists to support or refute this suggestion is a vivid demonstration of our poor understanding of the basic mechanisms of immunological development and the role of diet in its regulation. Ironically, the ability of milk to support the appropriate immune development of infant mammals argues that a scientific model with which to study the effect of diet on appropriate immune development is milk itself.

### 6.3.5. Mucins

Mucins are high molecular weight, highly glycosylated glycoproteins and unlike the secreted mucins of goblet cells, those in the MFGM are present as integral membrane components (Patton *et al.*, 1995). Milk fat globule mucins have been detected in many species, and are thought to be orthologues to human MUC1. Carbohydrates may constitute as much as 50% of the mass of the mucin molecule, and sialic acid in the terminal position of the oligosaccharide chains gives them a negative charge. Human MUC1 contains more galactose and *N*-acetylglucosamine, and less *N*-acetylgalactosamine and sialic acid than bovine MUC1. Carbohydrate epitopes on milk mucins have been probed using peanut, wheat germ and jack bean lectins, which detect the T-antigen ( $\beta$ -D-galactosyl (1-3)-*N*-acetyl-D-galactosamine), sialic acid and mannose, respectively. Both bovine and human milk mucins show T-antigen activity and sialic acid, whereas only bovine mucin seems to contain mannose, which is consistent with detection of *N*-glycosylation on this protein. The negative charge, which results from sialic acid, is thought to have functional implications in the prevention of globule coalescence, or in the regulation of the fat globule size (Patton *et al.*, 1995). Unlike human MUC1, which consists of a single polypeptide chain, bovine mucin is a heterodimer that is synthesized as a monomer, and cleaved during post-translational processing into a transmembrane domain and an extracellular glycosylated domain. Although the two segments remain bound noncovalently, bovine mucin is unstable to cooling and washing, and a portion of the extracellular glycosylated domain can be recovered in the serum phase when milk is cooled (Patton, 1999). The reason for this modification is not known.

As MUC1 extends from the epithelial and milk fat globule membranes, one function seems to be in providing protection for cells by acting as a physical barrier. Schrotten *et al.* (1992) found that components of the human MFGM bind to S-fimbriated *Escherichia coli*, and that mucin showed the highest activity. Furthermore, mucins with a molecular weight greater than 200 kDa, which were isolated from the feces of breast-fed but not from feces of formula-fed infants, prevented the attachment of the bacteria to the buccal epithelium. MFGM mucins also bind rotavirus and perhaps respiratory syncytial virus, and prevent replication (Yolken *et al.*, 1992). Deglycosylation of the mucin resulted in the loss of this activity.

To investigate the effect of gastrointestinal transit on MUC1, Patton (1994) analyzed fecal extracts of seven breast-fed and seven bottle-fed infants aged 20 days to 6 months using monoclonal antibodies directed against a tandem repeat of this mucin. Fragments of 200 kDa were detected in three of the seven breast-fed infants, but in none of those fed formula, which would be expected, as MUC1 is not present in infant formula. In a related study, Midtvedt *et al.* (1994) monitored mucin degradation activity in a group of 30 healthy Swedish children for 2 years. For those infants fed breast milk exclusively for at least 4 months, mucin degradation was initiated significantly later than in those who had received at least some formula. After one year, 21 children degraded mucin completely, while all children did so after 2 years of age.

The degradation of mucins requires the concerted action of proteases, esterases, sulfatases and glycosidases (Hoskins, 1992). These depolymerizing enzymes are not produced by the host, but rather by certain microbial residents at the entrance to the colon, representing about 1% of the total culturable fecal bacteria. They are Gram-positive, non-sporulating, obligately anaerobic, and non-pathogenic bacteria, and have been identified as *Ruminococcus torques*, *Ruminococcus gnavus* and *Bifidobacterium spp.* (Hoskins, 1992). With hog gastric mucin, which contains both the blood group A and H determinants (carbohydrate structures), as a substrate, ruminococci were able to degrade roughly 90% of mucin carbohydrate, while the bifidobacteria could degrade between 60 and 80%. The difference was attributed to the ability of ruminococci to cleave the terminal *N*-acetylgalactosamine A antigen. Further characterization of these isolates indicated that the glycosidases produced by the ruminococci were constitutively produced, released extracellularly, and were resistant to proteolysis, whereas, the glycosidases of the bifidobacteria are cell-bound. Co-culturing of both organisms led to increased overall oligosaccharide degradation and bacterial growth, due to the cooperative contribution of glycosidases, suggesting that this may be a characteristic feature of the gut microflora. Analysis of the spent medium indicated the presence of L-fucose, D-galactose,

*N*-acetylglucosamine, *N*-acetylgalactosamine, and the disaccharides lactosamine and galactose ( $\beta 1 \rightarrow 3$ ) *N*-acetylgalactosamine. Thus, the presence of certain strains of bacteria enables the cooperative degradation of glycoconjugates, which in turn increases the nutrient availability to other members of the gut microflora.

In the MFGM of human milk, mucin is associated with two other glycoproteins, butyrophilin and lactadherin. Yolken *et al.* (1992) found that a non-immunological fraction of human milk inhibited the replication of rotaviruses in tissue culture, and prevented the development of gastroenteritis in an animal model. Further characterization indicated that virus bound to mucin and to the associated 46 kD protein (identified as lactadherin). The biological functions of lactadherin may be related to its ability to interact physically with a wide variety of molecules.

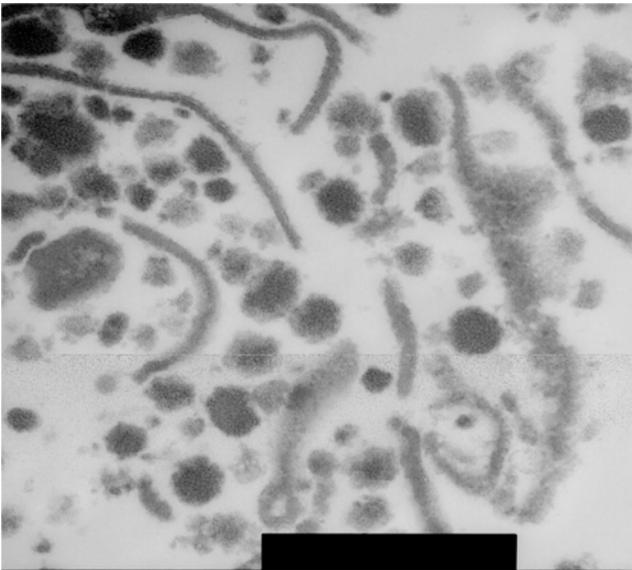
### 6.3.6. Xanthine Oxidoreductase

The protein product of a highly conserved housekeeping gene, Xanthine oxidoreductase (XOR), catalyses the oxidative degradation of the purine, xanthine, to uric acid and plays a role in the secretion of the milk fat globule (McManaman *et al.*, 2002). A hemizygous murine knockout of XOR is able to initiate, but not sustain, fat globule secretion (Vorbach *et al.*, 2002). Although the role of this protein in milk is not completely understood, it is thought to act outside of its characterized enzymatic role. XOR may have a functional role against pathogens, playing a part in the overall immune activity provided by milk (Vorbach *et al.*, 2003). The antimicrobial activity of XOR is a result of generating reactive oxygen and nitrogen species (ROS and RNS, respectively), and has been recognized for decades (Green and Pauli, 1943; Lipmann and Owen, 1943). Early studies used high enzyme concentrations, high oxygen tensions, and the effect was monitored by plate counts. The antimicrobial effects were attributed to the generation of the ROS, hydrogen peroxide. Using a strain of *Escherichia coli* that expressed a constitutive luminescent reporter, Hancock *et al.* (2002) showed that both bovine and human milk had bacteriocidal activity, and that it was reduced by boiling the milk, or by the addition of an XOR inhibitor, oxypurinol. The assay was conducted under hypoxic conditions, and was dependent on nitrite, presumably for the generation of RNS. When a little oxygen is present, nitric oxide (NO) and superoxide are formed, and can react to form the powerful bacteriocidal agent, peroxy-nitrate. As the  $K_M$  of XOR for nitrite is in the millimolar range and as some bacteria produce this concentration as a result of nitrate reductase, Hancock *et al.* (2002) suggested that the metabolism of the bacteria may result in their own undoing.

#### 6.4. Fractionation and Technological Significance of Milk Fat Globule Membrane Material

MFGM is found in significant quantities in dairy products such as cheese, cheese whey, butter and buttermilk. Milk fat globules are concentrated in cream and during the manufacture of butter the fat globules are disrupted mechanically. This process destabilizes the oil-in-water emulsion and results in two phases, fat granules and an aqueous phase rich in MFGM. The latter phase is traditionally called buttermilk, and is different in composition from the cultured product available commercially (Corredig and Dalgleish, 1998a). This byproduct of the industrial production of butter is enriched in MFGM. Figure 6.2 shows the presence in buttermilk of MFGM fragments and casein micelles (shown as elongated and round structures, respectively).

Increased recognition of the nutritional significance of components of the MFGM has led to a number of studies dedicated to the extraction and production of MFGM from buttermilk. Specific processes have been designed to obtain MFGM isolates free from other milk constituents, with the explicit objective of their use as bioactive and functional food ingredients. The differences in composition between isolates produced from different



**Figure 6.2.** Electron microscopy image of buttermilk sedimented by centrifugation. The sample was fixed with glutaraldehyde and post fixed with  $\text{OsO}_4$ . Bar = 0.70  $\mu\text{m}$ . Fragments of the MFGM are clearly visible.

sources and using alternative processes are not yet understood. However, understanding how different methodologies used to produce MFGM affect its composition will allow for fractions to be produced with unique functionalities, and could increase the value of buttermilk as a food ingredient.

#### 6.4.1. Effect of Processing on the Composition and Functionality of the MFGM

Agitation, cooling and ageing are just a few of the processes that cause changes to the composition of the MFGM post secretion (Evers, 2004). Table 6.1 summarizes some of the work reported in this area. It is important to note that the results of the analysis of the composition of MFGM may be affected by differences in the isolation method used. Despite careful handling of the fat globules in raw milk, supermolecular complexes between the MFGM proteins, Xanthine oxidoreductase, butyrophilin and adipophilin, are found in native milk fat globules. These large protein complexes fundamentally affect not only the biological functionality of the MFGM components, but also their stability and their changes with processing. While in fresh whole milk, the membrane consists mainly of phospholipids and MFGM proteins, on heating, the fat globule surface becomes coated with a layer of denatured proteins derived from the serum phase of milk (mainly whey proteins) (Dalgleish and Banks, 1991), and this effect is even more pronounced in cream (McPherson *et al.*, 1984). Very little is known about the

**Table 6.1.** Factors that affect the structure and composition of the MFGM

<b>Chemical/enzymatic</b>	
Loss of membrane components, ions, adsorption of milk plasma components, enzymatic activity	Anderson <i>et al.</i> (1972); Walstra (1983); McPherson and Kitchen (1983).
<b>Physiological</b>	
Diet, breed, stage of lactation	Anderson and Cawston (1975); Mondy and Keenan (1993).
<b>Handling and processing</b>	
Pumping, stirring, agitation	McPherson and Kitchen (1983).
Air	Van Boekel and Walstra (1989).
Cooling and ageing	Anderson <i>et al.</i> (1972). van Boekel and Walstra (1989); Sharma and Dalgleish (1993); Lee and Sherbon (2002).
Homogenization	Dalgleish and Banks (1991); McPherson <i>et al.</i> (1984); van Boekel and Walstra (1995); Corredig and Dalgleish (1996); Ye <i>et al.</i> (2002).
Heating	

changes that occur in the phospholipid fraction of MFGM with thermal processing.

When fat globules are heated in the absence of serum proteins, high molecular weight complexes form between butyrophilin and Xanthine oxidoreductase in less than 10 min at a temperature as low as 60°C (Ye *et al.*, 2002). In the presence of whey proteins, large amounts of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin associate with the MFGM (Corredig and Dalgleish, 1996; Ye *et al.*, 2004). Direct evidence of heat-induced covalent disulfide interactions between whey proteins and MFGM proteins can be obtained by electrophoresis and isoelectric focusing of the heated MFGM (Kim and Jiménez Flores, 1995; Corredig and Dalgleish, 1996; Ye *et al.*, 2002). The heat-induced formation of protein complexes on the surface of the MFGM may include denaturation of the individual proteins with the formation of aggregates containing MFGM proteins alone (butyrophilin, Xanthine oxidoreductase, PAS6/7) or MFGM proteins with whey proteins. The details of these reactions have been described by Ye *et al.* (2004).

Mechanical treatments such as agitation, pumping and high shear can cause changes in the composition of the MFGM, as well as changes in the size of the fat globules (McPherson and Kitchen, 1983). Homogenization is often employed to reduce the size of the fat globules, improve stability and delay creaming. During homogenization, the interfacial area increases significantly. Rearrangement of the original MFGM material occurs and considerably more protein is necessary to cover the newly formed interface. For this reason, casein micelles are adsorbed on the milk fat globules. This effect explains the observation that the fat globules in homogenized milk have a much higher protein load than untreated fat globules (Sharma and Dalgleish, 1993). Homogenization and heating are unit operations that are usually combined during milk processing. Differences in homogenization as well as the conditions of thermal treatment result in differences in the protein load on the MFGM surface and alter the ratio of whey proteins to caseins (Sharma and Dalgleish, 1993, 1994).

The formation of complexes between skim milk-derived proteins and MFGM proteins is of significance in milk processing. For example, the association of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin with the MFGM, which occurs with heat treatment, strongly affects the functional properties of MFGM isolates when used as ingredients in foods. Furthermore, the stability of oil-in-water emulsions prepared with MFGM isolates depends on the heat treatment of the original cream. The functional properties of the MFGM extracted from thermally-treated creams decrease as a result of heat treatment, even at a mild temperature (65°C) (Corredig and Dalgleish, 1998b).

### 6.4.2. Isolation of MFGM

Various bench-top extraction protocols have been developed to isolate MFGM for compositional analysis, and to facilitate an understanding of the secretion process of the fat globules. These investigations have provided strategies for the isolation of this material on a large scale. To achieve optimal isolation of the MFGM components (proteins and phospholipids), extractions should be carried out on freshly collected milk that has not been cooled (Mather, 2000). The unit operations used during the storage and processing of milk cause major changes to the MFGM, as has been demonstrated by the compositional analysis of the material extracted from various sources (for example, raw milk, heat-treated milk or cream).

In general, the common steps for the isolation of the MFGM include concentration of the fat globules (cream) using gravitational separation (often centrifugation), a series of washing steps to remove contaminants which adsorb loosely to the globules, and finally a step to destabilize the emulsion and separate the lipid and aqueous phases. These steps are general and common to bench-top and processing practices. The main difference between MFGM extracted in the laboratory versus that produced as a byproduct of industrial butter production is that the former is handled more carefully, and is washed and extracted using detergents, high speed centrifugation or salting out (McPherson *et al.*, 1984). A physical method such as strong agitation, churning or freeze-thawing is usually used to destabilize and rupture the MFGM.

Unlike the preparation obtained using laboratory isolation procedures, which normally include various steps to wash the fat globules to remove loosely associated contaminants, industrially processed buttermilk contains a large amount of serum proteins. Butter is prepared from cream containing of about 40% fat, and large amounts of caseins and whey proteins are still present in the aqueous phase. Buttermilk has physico-chemical properties similar to those of skim milk: it contains casein micelles with similar size and zeta-potential to those in skim milk, and a similar amount of total protein and protein soluble at a pH of 4.6 (O'Connell and Fox, 2000). In spite of the similarities in protein composition with skim milk, buttermilk is very distinct from any other dairy product and is recognized as a valuable source of phospholipids (Malmsten *et al.*, 1994; Sachedva and Buchheim, 1997). Sphingomyelin, phosphatidylcholine and phosphatidylethanolamine are present in an approximate ratio of 1:1:1 and represent most of the phospholipids in the MFGM (Parodi, 1997). This ratio makes the phospholipid extracts from the MFGM unique compared to the other sources of lecithin (egg and soy, for example). In spite of the availability of large amounts of these high-value components, buttermilk is still viewed as a

by-product and has very few applications as a value-added functional ingredient, mainly because to its low stability to oxidation.

A byproduct of cheese manufacture, whey cream, is the fat fraction separated from whey after removal of the curd and is also a good source of MFGM. Whey cream contains less skim milk-derived proteins, and MFGM can be recovered from the aqueous phase, which results from the destabilization of the fat globules. Whey cream, buttermilk as a by-product of the manufacture of whey butter, butter serum from the manufacture of anhydrous milk fat and buttermilk are less valuable than skim milk, because of the polyunsaturated fatty acids present, which are labile to oxidation. When compared to skim milk, buttermilk has high batch-to-batch variability, a characteristic that limits applications in food processing. All the processing steps involved in the production of buttermilk affect the interactions of the serum proteins with the MFGM. For this reason, a better understanding of the factors underlying these interactions, coupled with careful control of the processing parameters will be necessary to obtain products of consistent quality. Currently, this is often not the case for buttermilk and whey cream, as their processing history is not fully controlled. This presents an opportunity for research to resolve the inconsistencies found in these byproducts, by understanding how changes in the functionality of MFGM relate to the variations in manufacturing conditions.

Improvement of membrane separation technology has resulted in the isolation of MFGM-enriched material from commercially available products. A phospholipid-rich fraction can be extracted from whey (Boyd *et al.*, 1999) and buttermilk (Sachdeva and Buchheim, 1997) with a reported yield of 0.25 g of phospholipids/g of protein in buttermilk (Sachdeva and Buchheim, 1997). Microfiltration of whey derived from the Cheddar cheese process, using 0.2  $\mu\text{m}$  ceramic filters results in a fraction containing two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, and lesser amounts of phosphatidylinositol, phosphatidylserine, sphingomyelin and cerebrosides (Boyd *et al.*, 1999). The phospholipid fraction separated from the total lipids contains a larger proportion of mono- and polyunsaturated fatty acids (mainly oleic,  $\text{C}_{18:1}$  and linoleic,  $\text{C}_{18:2}$ ) compared to the total lipid and the neutral lipid fraction (Boyd *et al.*, 1999).

While in the laboratory it is possible to extract a MFGM fraction free from contaminants, although with some losses of membrane material during the washing steps, the isolation of MFGM from commercial dairy products is more challenging. In addition to the MFGM polypeptides, buttermilk and whey may contain significant amounts of components derived from skim milk (whey proteins, caseins). Only a few reports describe the separation of MFGM from casein micelles (Sachdeva and Buchheim, 1997; Corredig and Dalgleish, 1998c; Corredig *et al.*, 2003), which are comparable in size to the

MFGM fragments, and cannot be isolated by filtration only. The primary challenges are not in the optimization of MFGM fractionation with little protein contamination, but in scaling up of the technology. MFGM material can be separated from the other proteins and lactose in commercial buttermilk using centrifugation, by adding agents that disrupt the casein micelles and increase casein solubility (Corredig and Dalgleish, 1998c); however, this technique is not applicable to industrial-scale separation of MFGM. Different buttermilk fractions can be prepared by adsorbing the MFGM material on different types of biosilicates. These materials selectively extract the high molecular weight fraction of the MFGM from buttermilk, and have a higher binding affinity for phospholipids than neutral lipids (Fryksdale and Jiménez-Flores, 2001).

The caseins can be precipitated by treating buttermilk with rennet or citric acid. The phospholipid-rich serum can be filtered using 0.2  $\mu\text{m}$  membranes with good recovery of the MFGM fraction. The yield varies depending on coagulation conditions, especially pH (Sachdeva and Buchheim, 1997).

Microfiltration has shown the best potential to extract MFGM-rich fractions from buttermilk. Microfiltration with a small nominal pore-size membrane (0.1  $\mu\text{m}$ ) is often used to produce micellar casein, native phosphocaseinate, or to modify the ratio of caseins in milk (Mistry and Maubois, 1993; Pouliot *et al.*, 1994). Phospholipids can be concentrated using a 0.1  $\mu\text{m}$  membrane (Morin *et al.*, 2004). The use of reconstituted buttermilk, rather than fresh buttermilk, results in compositional differences in the final product (Morin *et al.*, 2004). However, microfiltration alone cannot achieve complete separation of MFGM lipids and proteins from caseins and whey proteins. Because of the comparable size of the components, retention of caseins during microfiltration of buttermilk can be reduced by increasing the amount of soluble casein (Corredig *et al.*, 2003). The separation of MFGM from skim milk-derived caseins can be achieved by filtering through 0.1  $\mu\text{m}$  pore size membrane after addition of sodium citrate to buttermilk. This optimizes the retention of fat and the high permeation of the caseins, and results in an enriched fraction from buttermilk containing about 80% of MFGM material (Corredig *et al.*, 2003). MFGM-enriched fractions obtained from buttermilk, although containing a significantly reduced amount of caseins, still contain a large amount of whey proteins (Corredig *et al.*, 2003). The lack of permeation of whey proteins, especially  $\beta$ -lactoglobulin and to a lesser extent  $\alpha$ -lactalbumin, shows the strong binding of whey proteins with MFGM components. The protein aggregates containing whey proteins are large enough to be retained during microfiltration and these complexes may be heat-induced polymers of whey proteins or, more likely, complexes with skim milk proteins (e.g.,  $\kappa$ -casein) or MFGM proteins.

A combination of microfiltration (with 0.8  $\mu\text{m}$  pore-size membranes) and supercritical fluid extraction has also been used to obtain MFGM isolates rich in phospholipids (Astaire *et al.*, 2003). Supercritical fluid extraction can be used to extract lipid and lipid-soluble materials from complex matrices. Analysis of the extracts obtained by first concentrating buttermilk by cross-flow microfiltration and then extracting the concentrate using supercritical fluid to remove the neutral lipids showed that the MFGM isolates contained a significantly reduced concentration of non-polar lipids and an increased concentration of polar lipids derived from the MFGM (Astaire *et al.*, 2003). Although it is possible to obtain a phospholipid-rich extract using this technology, it would be quite expensive and skim milk-derived material would still be present in significant quantities as contaminants.

#### **6.4.3. Application and Utilization of MFGM as a Functional Ingredient in Foods**

Although some components of buttermilk, butter serum or whey cream could, potentially, be isolated and marketed as dietary supplements, these products are also valuable when added to foods because of their functional properties as ingredients. Buttermilk has traditionally been considered to have superior functionality compared to skim milk in bakery and ice cream manufacture, because it contains MFGM material. For this reason, it is often used as an ingredient in food products because of its emulsifying properties and in low-fat products to improve flavor and texture. For example, the incorporation of ultrafiltered buttermilk into reduced-fat cheese improves the mouth-feel, body and meltability of the cheese. The addition of buttermilk to cheese milk increases the yield of low-fat Cheddar cheese by increasing the moisture content (Mistry *et al.*, 1996; Turcot *et al.*, 2001). The presence of MFGM has also been reported to improve the stability of processed foods (e.g., the addition of buttermilk enhances the heat stability of reconstituted evaporated milk) (Singh and Tokley, 1990).

Another suggested use for buttermilk solids as a value-added ingredient is to stabilize certain food matrices against lipid peroxidation (Wong and Kitts, 2003). Buttermilk solids are ineffective in delaying the onset of lipid oxidation, but reduce the severity of lipid oxidation during propagation. The concentrations tested were 0.1–0.2% in emulsion models, and at the same concentrations, whey proteins were less effective (Wong and Kitts, 2003). The antioxidant activity of buttermilk cannot be attributed solely to the total sulphhydryl content. In fact, heating reduces the total sulphhydryl content of buttermilk to levels lower than those reported for milk and whey (Taylor and Richardson, 1980).

MFGM fractions isolated from native milk fat globules have shown high surface activity. The measured interfacial tension on surfaces covered by native MFGM is similar to that covered by caseins (Chazelas *et al.*, 1995). These results confirmed other research reports on the emulsifying properties of untreated MFGM fractions. MFGM can be used as an emulsifier in reconstituted milk fat emulsions; MFGM can stabilize  $25 \times$  its mass of milk fat, forming droplets comparable in size to those in homogenized milk (Kanno, 1989; Kanno *et al.*, 1991). Model soy oil-in-water emulsions are also stabilized at neutral pH by MFGM prepared from untreated cream, but they are unstable at low pH (Corredig and Dalgleish, 1998c). The newly-formed oil droplets, covered by MFGM material, behave differently from emulsions stabilized by other milk proteins: no displacement occurs on the addition of small molecular-weight surfactants, and the addition of  $\beta$ -lactoglobulin or caseins after emulsification does not seem to affect the composition of the interface. In all these studies, the MFGM material was derived from cream, which had not been treated and no other protein was present in solution during emulsification.

The behavior of buttermilk as an ingredient in foods may be attributed in part to the presence of the MFGM, but it is predominantly determined by the presence of the skim milk-derived material, especially caseins (Corredig and Dalgleish, 1998a). Whey proteins and caseins constitute a large percentage of the total functional protein in commercial buttermilk. For this reason the functional properties of buttermilk result from the contribution of both skim milk-derived proteins and MFGM material. When buttermilk powder is used to prepare oil-in-water emulsions, caseins make up about 50% of the total protein adsorbed at the interface (Corredig and Dalgleish, 1998a). In these emulsions, the MFGM material does not show preferential adsorption over skim milk proteins and large aggregates are adsorbed at the interface. The behavior of oil-in-water emulsions prepared with buttermilk is different from that of skim milk, as in skim milk-stabilised emulsions less protein is needed to stabilize the oil droplet surfaces than in buttermilk-stabilised emulsions. In addition, preferential adsorption of the caseins over the whey proteins takes place in homogenized skim milk but not in buttermilk (Walstra and Oortwijn, 1982).

The functional properties of the MFGM fragments present in buttermilk cannot be related directly to those of MFGM extracted from untreated cream, because the processing history of MFGM strongly influences its functional properties. Treatment at a temperature as low as  $60^{\circ}\text{C}$  affects the emulsifying properties and solubility of MFGM isolates. MFGM fractions prepared from various heat-treated creams contain considerable amounts of associated whey proteins, and their emulsifying properties are poor compared to those of MFGM material extracted from unpasteurized

cream (Corredig and Dalgleish, 1998b). The decrease in emulsifying properties and solubility are related directly to the amount of whey protein associated with the MFGM and the temperature applied (Corredig and Dalgleish, 1998b). Such changes in functionality of the MFGM with heat treatment and processing need to be understood to explain the variability of the sources of buttermilk available commercially. The functionality of MFGM prepared from heat-treated cream can be modified by proteolysis with trypsin or chymotrypsin. When adsorbed onto oil droplets, the MFGM isolated from treated buttermilk (commercially available) is more accessible to enzymatic treatment than MFGM prepared from untreated cream (Corredig and Dalgleish, 1997). In addition, the emulsifying properties of the MFGM preparations can be improved by treatment with trypsin or chymotrypsin.

#### **6.4.4. Conclusions and Future Research Directions**

Living mammals are the progeny of those for whom lactation was successful. The genetic legacy of the relentless selective pressure on milk as a nourishing food is encoded in the genes responsible for milk production (i.e., the milk genome). From the compositional information, which exists on milk from different species, at different stages of lactation and in response to various external inputs, it is clear that this biomaterial is highly dynamic. To be successful in competitive habitats, lactation must be effective in providing nourishment and protection for the neonate while not excessively taxing the mother's resources. As is true for much of molecular evolution, many of the components of milk have been co-opted from other functions in biology, and in most cases it is not clear why. Yet, it can be argued that components that provided no benefit, or which were no longer needed, would tend to be lost over time. Against this background of evolutionary selection, comparative milk genomics becomes a relevant scientific endeavour for beneficial nutritional bioactivities. The challenge to the nutritional and food sciences is to understand the molecular basis of these benefits. Understanding how milk functions will ultimately allow similar benefits to be extended to other foods and other consumers.

The arrival of the genomic age offers a range of new tools for scientists to approach these complex biological questions. The assembly of vast nucleotide and protein sequence databases, together with the concomitant development of the bioinformatic tools to analyze them, gives researchers a unique window into the mammalian genome information space. As the first pass sequencing of the human genome is now largely completed, work has begun to annotate all the genes for their endogenous function. The inherent power of this approach is that the databases are additive, and integrative. As proteins are

identified as constituents of the MFGM, the following information should contribute to an understanding of their function, such as the primary sequence, domain structure, and function in other cell types. Identification of the genetic basis for milk components specific to particular functional motifs, coupled with the physiological knowledge of their mechanisms of action will give insight as to how these components are functioning.

The MFGM contains a large amount of components that contribute to the compositional diversity in milk. In addition, numerous MFGM constituents have recognized bioactivity. While research continues to reveal evidence of the relationship between dietary consumption of MFGM and enhanced health, more research is needed to understand the functionality of the various components present in this material. The process engineering of dairy streams could be modified to take advantage of the MFGM if such information was available. For example, at present, high heat is applied to cream for butter processing; if a less, severe heat treatment was used, highly functional MFGM material would be available, which could be marketed as a value-added ingredient for its nutritional functionality as well as its processing functionality (e.g., as an ingredient that improves texture and mouth-feel).

Only a few reports are available on the preparation of MFGM from commercially available sources and the opportunities to exploit fully the utilization of MFGM as a functional material are so far limited by the lack of available products and commercially feasible preparation methods. The development of methods for the extraction of MFGM from buttermilk through microfiltration may increase the opportunity to produce this ingredient on a commercial scale. On the other hand, before the economics of such processes can be appreciated, the unique functionality of MFGM isolates needs to be understood better.

The conditions to which fat globules are subjected during processing compromises the native structure and composition of the MFGM and are likely to have dramatic consequences on its functionality. Hence, greater exploitation of the value of MFGM may require redesigning of some of the dairy processes to which the globules are subjected.

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# Crystallization and Rheological Properties of Milk Fat

A.J. Wright and A.G. Marangoni

## 7.1. Introduction

The crystallization behavior and rheological properties of milk fat are extremely important to the processing and texture of dairy and dairy-based foods. For example, the crystal network structure of butter depends on its composition and the crystallization behavior of the milk fat present. In turn, these properties determine the end use applications, spreadability, mouth-feel, appearance and even the taste of butter. In this chapter we will review current understanding of milk fat crystallization, structure and mechanical properties. Manipulation strategies for altering the properties of milk fat, including improved butter consistency, will also be discussed. Finally, three case studies establishing the links between milk fat composition, crystallization, structure, and rheological properties will be presented.

## 7.2. Crystallization of Milk Fat

### 7.2.1. Introduction

The principal determinant of butter consistency is the ratio of solid to liquid fat (Rohm and Weidinger, 1993). Therefore, the extent of crystallization is critical to the texture of butter. Milk fat is composed of literally hundreds of unique and varied triacylglycerol (TAG) species (Jensen *et al.*, 1991). This results in milk fat having complicated crystallization, melting, and rheological behaviour (Mulder, 1953; Hannewijk and Haighton, 1957).

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

Both compositional and processing conditions influence crystallization. Composition varies with season, region and breed of animal (deMan and Wood, 1958a). For example, butter produced during the winter tends to have a higher level of palmitic and less oleic acid than butter produced during the summer. This results in a lower iodine value (IV) for winter butter (~36 versus ~30) and a firmer consistency (Prentice, 1972). The texture of butter depends on many interrelated parameters, although fatty acid composition and positional distribution are very important because they determine melting temperature (Kleyn, 1992). Fatty acid chain length and IV account for much of the variation in butter firmness. A change in IV of 3% can effect a 50% change in firmness (Hayakawa *et al.*, 1986).

Processing conditions also impact on the crystallization and ultimately the rheology of milk fat. For example, continuously churned butter is typically harder and less spreadable than conventionally batch-churned butter (deMan and Wood, 1958a). The external factors that can influence the crystallization of milk fat include temperature (Tverdokhle and Gulyaev-Zaitsev, 1966; Herrera and Hartel, 2000a; van Aken and Visser, 2000), cooling rate (deMan, 1964; Schaap and Rutten, 1976; Herrera and Hartel, 2000a), scale of operation (Saxer and Fischer, 1983), agitation (Black, 1975; Schaap and Rutten, 1976; Keogh and Higgins, 1986; Grall and Hartel, 1992), and storage conditions (Mortensen and Danmark, 1982a; Precht, 1988). The state of fat dispersal also has an influence on crystallization behavior (Mulder and Walstra, 1974). Differences between TAG crystallization in the bulk and emulsified states have been noted by several groups (Mulder, 1953; Mulder and Walstra, 1974; Phipps, 1957; Skoda and van den Tempel, 1963; Walstra, 1975b; Walstra and van Berestejn 1975a; van Vliet and Klock, 1995). In general, finely dispersed fats require high supercooling and have a low crystallization rate. This is explained by differences in nucleation between the two systems (Walstra and van Berestejn, 1975a). The behavior of butter will lie somewhere between that of globular and continuous fat (Mulder and Walstra, 1974).

Crystallization refers to the change from a liquid to a solid state and is an exothermic process. It involves nucleation, crystal growth and crystal rearrangements. Crystallization is influenced by both kinetic and thermodynamic factors (Sato *et al.*, 1989). The kinetics of milk fat crystallization have been reviewed by many groups, including Grishenko (1959), deMan (1963a), Tverdokhle and Gulyaev-Zaitsev (1966), Mulder and Walstra (1974), Walstra and Jenness (1984) and Walstra *et al.* (1994). Kinetic parameters involved in milk fat crystallization include the clustering of molecules, molecular adsorption, diffusion, solvation/desolvation and conformational rearrangements. The surface-melt interfacial free energy and the crystallization temperature are

thermodynamic parameters, which influence crystallization (Sato *et al.*, 1989). The temperature at which a fat is crystallized is a major determinant of the reaction kinetics and resultant structure. Temperature determines the extent of supersaturation and represents the driving force for nucleation.

### 7.2.2. Nucleation of Milk Fat

When a fat is cooled to a temperature below its melting point, the molecules are “supercooled”. Supercooling is equivalent to supersaturation and is the thermodynamic driving force for crystallization to occur. In this non-equilibrium state, molecules begin to aggregate into tiny clusters (i.e., embryos), which continuously form and dissolve until some critical size is reached. At this point, the cluster is referred to as a nucleus (Garside, 1987). Nuclei form only when the energy associated with the latent heat of crystallization is greater than the energy needed to overcome the increase in solid surface area (Timms, 1995). The critical radius at which nuclei are stable depends on temperature. At low temperatures, smaller clusters are stable because of decreases in the solubility of TAGs and increases in the free energy change (Lawler and Dimick, 1998). Larsson (1994) suggested that, rather than forming spherical nuclei, TAG molecules probably align laterally and then arrange into bilayers that reach a critical size.

Three types of nucleation are generally discussed for fats: primary homogeneous, primary heterogeneous, and secondary. Homogeneous nucleation occurs in pure solutions in the absence of foreign materials or interfaces. In milk fat, this type of nucleation is very rare. It can occur only at very high degrees of supercooling, at temperatures near or below 0°C (Walstra and van Beresteyn, 1975a). More often, nucleation is heterogeneous in nature. Heterogeneous nucleation is catalyzed by the presence of foreign particles or interfaces. It requires a much lower level of supercooling (van den Tempel, 1968; Garside, 1987; Boistelle, 1988). Secondary nucleation is very important in milk fat crystallization (Walstra, 1998). During secondary nucleation, nuclei form upon contact with existing crystals (Larsson, 1994). This is promoted by agitation, which breaks apart existing crystals and increases the solid surface area.

Differences in nucleation explain the differences observed between crystallization in the bulk and emulsified states. In bulk fats, only a small number of nuclei are needed to induce crystallization. However, when the same fat is emulsified, each fat droplet must contain a nucleus or impurity in order to crystallize, the probability of which is low. As a result, the emulsified fat requires more supercooling (i.e., to a lower temperature) in order to nucleate (Walstra *et al.*, 1994).

### 7.2.3. Growth of Milk Fat Crystals

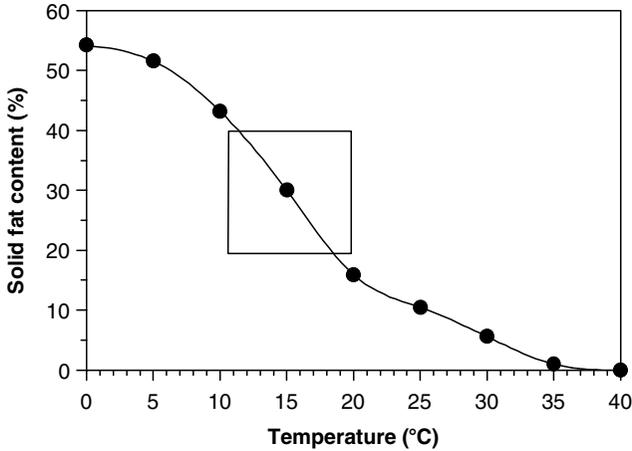
The rate of crystal growth is determined by the degree of supersaturation, the rate of molecular diffusion to the crystal surface, and the time required for TAG molecules to fit into the growing crystal lattice (Mulder and Walstra, 1974; Walstra, 1987). Compared to nucleation, the driving force required for crystal growth is relatively low (Sato *et al.*, 1989). However, in a multicomponent fat, the supersaturation for each TAG is small (Walstra, 1998). This fact, combined with competition between similar molecules for the same sites in a crystal lattice, means that milk fat crystallization is especially slow (Skoda and van den Tempel, 1967; Knoester *et al.*, 1968; Grall and Hartel, 1992).

Melt viscosity also has a significant effect on crystal growth rate. It limits both molecular diffusion and the dissipation of the latent heat of crystallization away from a growing crystal (Walstra *et al.*, 1994). Growth rate is inversely proportional to melt viscosity, which tends to increase with decreasing temperature (Lawler and Dimick, 1998). Shearing forces, too, influence fat crystallization. The effects of shear include increased secondary nucleation as a result of crystal fracturing and perhaps ease of nucleation because of the parallel alignment of TAGs (Stapley *et al.*, 1999). Shear also enhances both mass and heat transfer. During milk fat fractionation, shear was found to influence both the composition and the structure of the filtered crystals (Breitschuh, 1998; Breitschuh and Windhab, 1998).

### 7.2.4. Crystallization, Melting and Mixed Crystal Formation

The overall dropping temperature of milk fat is approximately 34°C. However, because milk fat contains over 400 different TAGs (Jensen *et al.*, 1991), each with its own melting temperature, it demonstrates a wide melting range as opposed to a distinct melting temperature (Walstra *et al.*, 1994). The TAGs in milk fat have a melting range between -40 and 40°C. This results in a wide range of plasticity where both solid and liquid fat are present as shown in Figure 7.1.

When TAGs in the liquid state are mixed, no changes in heat or volume are observed (Walstra *et al.*, 1994). However, ideal behavior is not observed in the solid phase of milk fat (Timms, 1984; Walstra *et al.*, 1994). As a result, the melting curve of milk fat does not equal the sum of its component TAGs (Walstra *et al.*, 1994). Mulder (1953) proposed the theory of mixed crystal formation to explain the complex crystallization behavior of milk fat. Mixed or compound crystals contain more than one molecular species (Rossell, 1967; Mulder and Walstra, 1974). Mixed crystals form in natural fats, like milk fat, which are complex mixtures of TAGs (Mulder, 1953; Sherbon 1974; Walstra and van Beresteyn, 1975b; Timms, 1980;



**Figure 7.1.** Solid fat content (SFC) of milk fat as a function of temperature. The box indicates the temperature region (i.e., 11–20°C) in which SFC ranges from 20 to 40% and good spreadability is expected.

Walstra *et al.*, 1994; Breitschuh and Windhab, 1998; Marangoni and Lencki, 1998). Mixed crystals have a lower density and a lower enthalpy of fusion than pure crystals of the same polymorph. They also tend to rearrange slowly into purer crystals (Walstra, 1987).

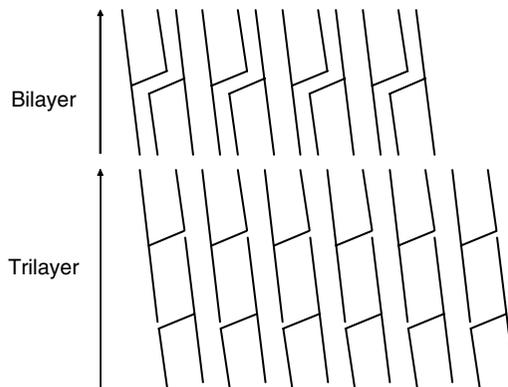
The likelihood of compound crystallization increases when the molecular species are similar in shape, size and properties (Walstra, 1987). It also increases during crystallization at a low temperature because more TAGs are supersaturated and the supercooling for each TAG is higher (Walstra *et al.*, 1994). Mixed crystals also tend to form more readily in less stable polymorphic forms because molecular packing is not very dense (Mulder and Walstra, 1974). Slow and stepwise cooling of milk fat gives a lower solid fat content (SFC) than rapid and direct cooling. The mixed crystal concept explains this observation and also the observation that it is difficult to establish equilibrium in milk fat (Mulder and Walstra, 1974). Crystal rearrangements including segregation into more pure crystals and Ostwald ripening can occur indefinitely.

Several studies have explored the phase behavior of milk fat and its fractions (Mulder, 1953; Timms, 1980, 1984; Marangoni and Lencki, 1998). Milk fat composition is often discussed in terms of groups or fractions of TAGs, which are chemically and physically distinct (Timms, 1980; Bornaz *et al.*, 1993; Marangoni and Lencki, 1998). For example, saturated and monounsaturated TAGs account for 65 mol% of the TAGs in milk fat

(Gresti *et al.*, 1993). TAG fractions are typically distinguished from each other on the basis of melting behavior. Accordingly, there are three main fractions of TAGs, the low-melting, middle-melting and high-melting fractions (LMF, MMF and HMF, respectively). These fractions correspond to the three endothermic peaks observed in milk fat by differential scanning calorimetry (Timms, 1980). LMF is liquid at room temperature owing to its high content of long-chain unsaturated and short-chain saturated fatty acids. On the other hand, HMF has a melting temperature greater than 50°C and is enriched in long-chain saturated fatty acids. The HMF has a much lower content of long-chain unsaturated and short-chain saturated fatty acids. The MMF is characterized by an intermediate melting temperature range of 35–40°C (Timms, 1980). In milk fat, the higher-melting TAGs tend to dissolve in the lower-melting species (Walstra *et al.*, 1994). Also, in the solid phase, incomplete miscibility is observed because of the wide range of TAGs present (Timms, 1984). These factors complicate the fractionation of milk fat into different groups of TAGs.

### 7.2.5 Polytypism and Polymorphism

When lipids crystallize, variations in terms of how the TAGs stack and how the fatty acids pack are possible (Sato *et al.*, 1989). Polytypism refers to the different stacking directions of the TAG lamellae in fats. The lamellae layer thickness (or d-spacing) depends on both the length of the TAG molecules and the angle of tilt between the chain axis and the basal lamellar plane (Lawler and Dimick, 1998). Polytypism is indicated with a  $-2$  or  $-3$  designation following the polymorph type for a bilayer or trilayer TAG arrangement (shown in Figure 7.2), respectively (Lawler and Dimick,



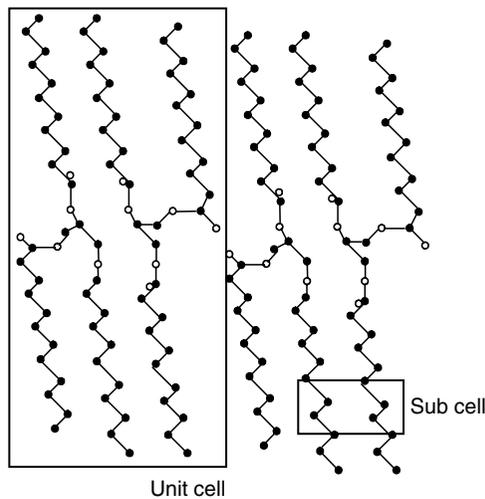
**Figure 7.2.** Polytypism in crystalline triacyl glycerols: Bilayer and trilayer arrangements.

1998). The bilayer arrangement of fatty acid chains is the most common packing structure for natural fats, including milk fat. However, with quench cooling to  $-8^{\circ}\text{C}$ , Lopez *et al.* (2001) found evidence of the coexistence of both the bilayer and trilayer lamellar structures in milk fat.

Polymorphism refers to the situation in which materials of the same chemical composition possess different sub-cell packings in the solid state (Small, 1986). Figure 7.3 compares the level of structure for the unit cell and sub-cell in crystalline fats. Polymorphism arises from both variations in the tilt of TAG molecules in a bilayer and from variations in the hydrocarbon chain packing (Larsson, 1994).

Polymorphism explains the multiple melting phenomena, which are observed in fats. Mulder (1953) reported that if milk fat is cooled rapidly, two melting temperatures are observed subsequently. deMan (1963b) used X-ray diffraction (XRD) to identify the  $\alpha$ ,  $\beta'$  and  $\beta$  polymorphs in milk fat. Different packing modes give rise to differences in density, melting temperature, and heat of fusion for the polymorphs. The melting temperature of  $\alpha$ ,  $\beta'$  and  $\beta$  polymorphs in milk fat are reported to be  $22$ ,  $30$  and  $35^{\circ}\text{C}$ , respectively (van Berestejn, 1972). More recently, Ten Grotenhuis *et al.* (1999) reported the clear point of the  $\alpha$ -crystal in milk fat to be  $20^{\circ}\text{C}$ .

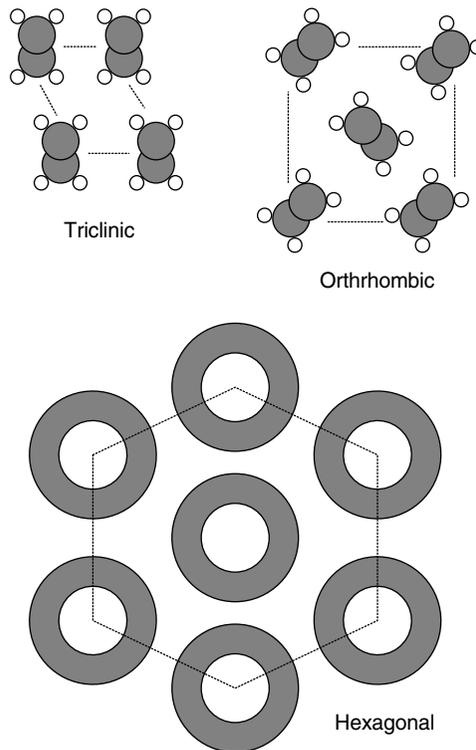
The  $\alpha$ ,  $\beta'$  and  $\beta$  polymorphs have characteristic XRD short spacings of  $0.415$  nm,  $0.38$  nm and  $0.42$  nm and  $0.46$  nm, respectively (Small, 1986; D'Souza *et al.*, 1990). The  $\alpha$ -sub-cell has an hexagonal arrangement in which



**Figure 7.3.** Unit cell and sub-cell levels of structure in crystalline triacyl glycerols.

the fatty acid chains show disorder and rotational mobility along the hydrocarbon chain axes (Larsson, 1994). The  $\beta'$ -polymorph has a more fixed sub cell arrangement referred to as orthorhombic perpendicular, while the  $\beta$ -crystal has the most dense packing and is triclinic (Abrahamsson *et al.*, 1978; Gunstone *et al.*, 1986; Small, 1986). The hexagonal, orthorhombic perpendicular, and triclinic sub-cell packings for the  $\alpha$ ,  $\beta'$  and  $\beta$  polymorphs are shown in Figure 7.4.

The  $\beta$  polymorph is the most thermodynamically stable. It has the highest melting point and is therefore the least soluble in a melt at a given temperature below its melting point. Despite this, nucleation for the  $\alpha$ -polymorph is favored. Although the  $\alpha$ -crystal is less stable, it has a lower crystal-melt interfacial tension and lower heat of crystallization than the  $\beta'$ - and  $\beta$ -polymorphs (Timms, 1995). Nucleation in milk fat typically



**Figure 7.4.** Arrangement of carbon and hydrogen atoms in fatty acids with hexagonal, orthorhombic perpendicular and triclinic crystals, for the  $\alpha$ ,  $\beta'$  and  $\beta$  polymorphs, respectively.

occurs in the  $\alpha$ -form (Walstra, 1987) because nucleation and crystal growth rates are higher than for the  $\beta$  and  $\beta'$  polymorphs (Sato *et al.*, 1989). Fat polymorphism has practical consequences for product quality and functionality. Hoerr (1960) described the  $\alpha$ -form in fats as very thin crystals less than several microns in size and the  $\beta'$ -polymorph as long needle shapes less than  $5\ \mu\text{m}$ . In comparison,  $\beta$ -crystals were described as much larger and having a plate-like morphology (Hoerr, 1960).

Like most multi-component fats, milk fat exhibits monotropic polymorphism. Only one polymorph is stable (Bailey, 1951). Molecular rearrangements result in polymorphic transformations from the less to the more thermodynamically stable forms (Hagemann, 1988). Figure 7.5 shows the polymorphic transformations that occur in edible fats.

Milk fat is considered to be a  $\beta'$ -fat. Even after prolonged storage, the majority of the crystals in the fat remains in the  $\beta'$  form (Timms, 1979). Although the  $\beta$ -polymorph has been identified in milk fat in some studies (deMan, 1963a; Woodrow and deMan, 1968; Timms, 1979), other researchers have found no evidence of it (Ten Grotenhuis *et al.*, 1999). Ten Grotenhuis *et al.* (1999) detected the  $\alpha$ -modifications and  $\beta'$ -modifications and with quench cooling to extremely low temperatures, another short-lived modification referred to as  $\gamma$ . The  $\gamma$ -form was referred to as sub- $\alpha$  because its melting temperature and stability are lower than  $\alpha$ . The same form has also been referred to as  $\beta'_2$  because it has an orthorhombic structure like the  $\beta'$ -form. The  $\gamma$ -polymorph forms in milk fat only at high cooling rates and has a clear point of approximately  $-8^\circ\text{C}$  (Ten Grotenhuis *et al.*, 1999).

Although the  $\alpha$ -polymorph is meta-stable, it can have a relatively long lifetime in milk fat at low temperatures compared to other fats (Walstra *et al.*, 1994). The  $\alpha$ -crystal may be stabilized by the formation of compound crystals in milk fat (Walstra *et al.*, 1999). A consequence of both polymorphism and mixed crystal formation in milk fat is that the material is rarely at equilibrium (Walstra *et al.*, 1994).

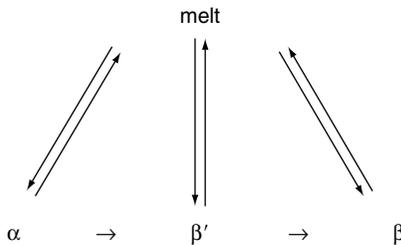


Figure 7.5. Polymorphic transformations in fats.

## **7.3. Structure and Rheology of Fat Crystal Networks**

### **7.3.1. Milk Fat and Butter Crystal Networks**

The underlying structures of both milk fat and butter determine their rheological and textural properties. The first comprehensive review of butter structure was prepared by King (1964). More recently, deMan and Beers (1987), Juriaanse and Heertje (1988), Precht (1988) and Heertje (1993) reviewed the literature on the structure of milk fat and butter. Holcomb (1991) compiled a list of references pertaining to the structure and rheological properties of dairy products. When cream is churned, phase inversion occurs and butter is formed. The multi-phase emulsion consists of a continuous oil phase containing crystal aggregates along with intact and fractured fat globules (Kalab, 1985; Juriaanse and Heertje, 1988). Although butter is an emulsion, its rheological properties are similar to those of milk fat. The aqueous phase is distributed in droplets and may contribute to the viscosity of the system (Prentice, 1992).

During crystallization, fat crystals aggregate into three-dimensional networks as a result of Brownian motion and van der Waals forces (van den Tempel, 1961). In both milk fat and butter, underlying fat crystal networks give these materials their structural integrity (Haighton, 1963; Knoop, 1964). Their yield value and viscoelastic behavior are a result of the crystal networks, which are intimately associated with the remaining liquid oil phase (deMan and Beers, 1987). The rheological properties of milk fat are discussed in a subsequent section.

### **7.3.2. Methods used to Determine the Rheological Properties of Milk Fat**

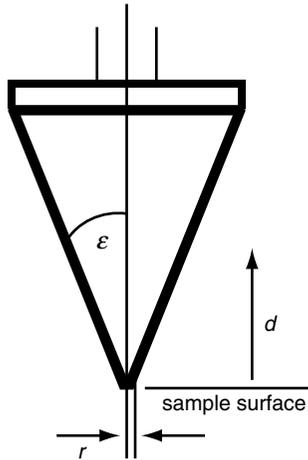
Most of the methods used to characterize the rheological behavior of butter are empirical and attempt to imitate certain sensory perceptions. They typically involve penetrometry, extrusion or sectility tests (Prentice, 1972). In these tests, the structure of the material is destroyed in order to probe its response to an applied stress or deformation. These methods mostly serve a quality control function. Their results provide an index of consistency to adjust milk-blending operations or to regulate a step in the butter-making process. While the results have practical significance, they often have no theoretical basis. Therefore, attempts have also been made to study the intrinsic properties of plastic fats. In many such cases, small deformation tests, in which the structure of the sample remains intact have been used to probe milk fat rheology.

### 7.3.2.1. Large Deformation Rheological Testing of Milk Fat and Butter

#### 7.3.2.1.1. Penetrometry-based Testing

Penetrometry has been the most common method used to evaluate butter texture (deMan and Beers, 1987). In penetrometry, either the depth to which a penetrating body (a cone, needle or sphere) falls during a specified length of time, or the rate at which the body falls is measured (Sherman, 1976). The technique was first applied by placing a steel rod above a sample and weighing it down until it rapidly penetrated the fat (Brulle, 1893). Today, cone penetrometry under a constant load remains the most widely used instrumental method to evaluate the texture of butter. Figure 7.6 shows the shape of a typical cone used in penetrometry testing.

Cone penetrometry has the advantages of being simple and economical to use. Also, its results correlate well with testing by sensory panels (Dixon, 1974; Rousseau and Marangoni, 1999). In addition, standardized tests and commercial standards of design are available. The most widely used method is that of the American Oil Chemists Society (AOCS) (*Cc 16-60*) (AOCS, 1960). According to this method, the depth ( $d$ ) (in increments of 0.1 mm) to which the cone penetrates the sample is read (AOCS, 1989). This depth is an indicator of consistency and can be related to some structural parameter of the material. Penetrometry results, for example, are often translated into “spreadability” or “hardness” values. For example, spreadability ( $S$ ), as



**Figure 7.6.** Shape of a typical cone geometry used in penetrometry studies on milk fat. The depth to which the cone penetrates the sample ( $d$ ) is used to calculate “hardness” and “yield” values.  $\epsilon$  is the cone angle/2 and  $r$  is the radius of the flat tip of the cone.

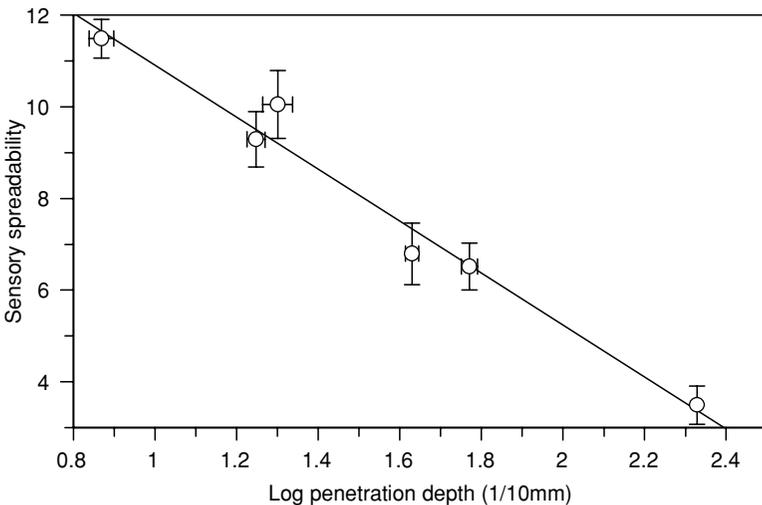
determined by a sensory panel, is correlated with penetration depth ( $d$ ) according to Equation 1:

$$S = A + \log(d) \quad (1)$$

Figure 7.7 shows that good agreement was found between spreadability as determined by a sensory panel and the depth of cone penetration as determined by the AOCS *method Cc 16-60* (Rousseau and Marangoni, 1999).

Vasic and deMan (1968) defined hardness ( $H$ ) as the ratio of load to the area of the impression made by the penetrometer. This parameter was explained as “the cone will sink into the fat until the stress exerted by the increasing contact surface of the cone is balanced by the hardness of the fat” (deMan, 1983). Vasic and deMan (1968) defined fat hardness in a similar way to the Brinell hardness used in metallurgy (Tabor, 1948). The relationship between the applied force load ( $P$ ), hardness ( $H$ ), half cone angle ( $\varepsilon$ ), radius of the flat tip of the cone ( $r$ ), penetration impression area ( $A_{\text{imp}}$ ) and depth ( $d$ ) for the cone in Figure 7.6 is given by Equation 2 (Vasic and deMan, 1968):

$$H = \frac{P}{A_{\text{imp}}} = \frac{P}{\frac{\pi d}{\cos(\varepsilon)}[2r + d \tan(\varepsilon)] + \pi r^2} \quad (2)$$



**Figure 7.7.** Correlation between milk fat spreadability as determined by a sensory panel and depth of cone penetration determined according to AOCS *method Cc 16-60* (Rousseau and Marangoni, 1999).

Converting penetration depth to hardness has the advantage of normalizing consistency values so that they are less dependent on the penetration load. This is the rationale behind hardness testing in metallurgy. In these cases, the contact pressure as defined by hardness in Equation 2 is used to deduce the yield stress of a material (Tabor, 1996). However, the yield stress is the resistance to an applied shear stress, but it is not the only resistance to a penetrating body. The elastic properties of a fat, and the coefficient of friction between the cone and the fat sample will also impede the penetration of the cone (Tabor, 1948). Kruisher *et al.* (1938) tried to eliminate friction effects and advocated the use of a flat circular penetrometer with concave sides.

Constant-speed cone penetrometry has also been used to measure the elastic properties of fats (Diener and Heldman, 1968; Briscoe and Sebastian, 1993). It measures the force required to drive a penetrating body into a sample at a constant speed (Kruisher *et al.*, 1938; Tanaka *et al.*, 1971). This approach allows for better control over penetration depth and allows for multiple hardness values (with varying penetration depth) to be calculated. Because the load-deformation response can also be monitored during unloading of the sample, additional rheological parameters can be derived with constant-speed penetrometry (Glenn and Johnston, 1992; Page, 1996).

Much more effort has gone into relating hardness value to the yield stress of fats than to their elastic properties. For example, the International Dairy Federation proposed (Walstra, 1980) that penetration depth be converted to an “apparent yield stress” (AYS) for sharp-ended cones according to the equation:

$$AYS = \frac{P}{A_{\text{proj}}} = \frac{gw}{\pi d^2 \tan^2(\varepsilon)} \quad (3)$$

where  $g$  is acceleration due to gravity,  $w$  is the weight of the cone assembly,  $\varepsilon$  refers to the cone half-angle (as shown in Figure 7.6) and  $d$  is the penetration depth. The hardness defined in Equation 2 and the AYS in Equation 3 are related to each other simply by a  $\sin(\varepsilon)$  term because  $A_{\text{proj}}$ , the projected area, is used in Equation 3 rather than the impression area (Mohr and Wellm, 1948). A “yield value” ( $C$ ) can also be determined for a smooth-angle cone according to Equation 4:

$$C = \frac{Kw}{d^n} \quad (4)$$

where  $w$  is the weight of the cone,  $d$  is the penetration depth and  $K$  is a factor depending on the cone angle (Haighton, 1959). The constant,  $n$ , was found

empirically to approximate 2, but researchers have since found that it varies with sample structure (deMan, 1976; Mortensen and Danmark, 1981). A value of 1.6 is often used for plastic fats.

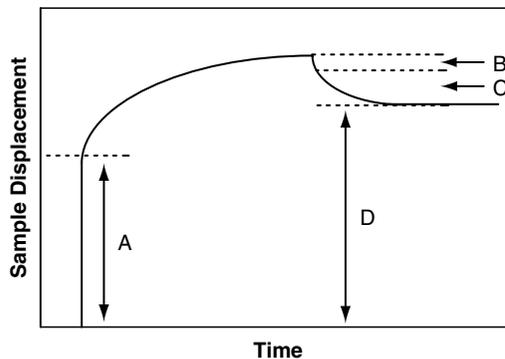
Spreadability is another important parameter of butter texture. A spreadability index ( $S$ ) can be calculated from the “yield stress” value obtained for butter before and after working using a constant-weight penetrometer, as shown in Equation (5), where  $f_u$  and  $f_w$  are the “yield stress” values before and after working respectively (Haighton, 1965).

$$S = f_u - 0.75[f_u - f_w] \quad (5)$$

### 7.3.2.1.2. Compression-based Testing

Compression testing is also useful for evaluating the technological and end-use properties of milk fat and butter (Davis, 1937; Scott-Blair, 1938; Dolby, 1941a; Mohr and Wellm, 1948). In this approach, a uniform stress is applied to the top and bottom of a sample (typically a cylinder or prism) placed between two flat plates. Because of the uniform stress field, it is easier to extract rheological information than in penetrometry.

Compression testing is often performed in the creep mode. A constant load is applied to the upper plate and then the deformation (i.e., how the sample changes) is recorded over time. Typical results of a creep test are shown in Figure 7.8; compliance is plotted as a function of time.

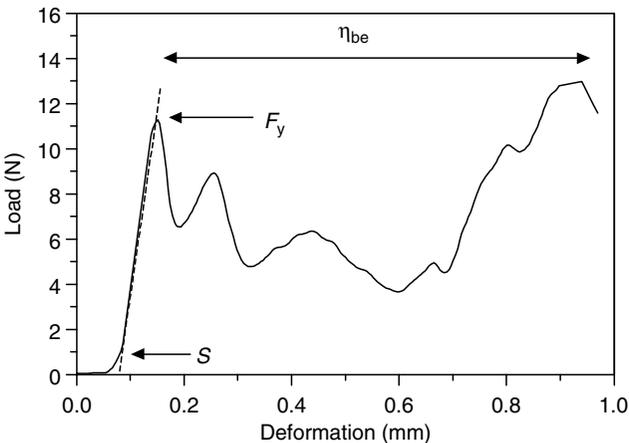


**Figure 7.8.** Typical creep curve for a plastic fat. A: instantaneous deformation upon loading; B: instantaneous sample recovery upon unloading; C: time-dependent recovery of sample; D: permanent sample deformation (adapted from deMan and Beers, 1987).

A and D indicate the two parameters most commonly extracted from a creep curve. A represents the instantaneous elastic compliance and can be used to calculate an elastic modulus. D represents the limiting viscosity, which is related to the reciprocal of the slope. In some cases, parameters from creep testing have been related to molecular mechanisms (Shama and Sherman, 1970; Davis, 1973; deMan *et al.*, 1985). The parameters have also been correlated with hardness and spreadability (Scott-Blair, 1938).

Compression testing can also be carried out at a constant speed. In this case, the applied load, in addition to the deformation of the sample, is monitored. The load-deformation curve for a sample of milk fat (crystallized for 24 hours at 5°C) is shown in Figure 7.9.

Various parameters can be derived from the curve, including a compressive modulus (based on initial stiffness ( $S$ ) and sample dimensions) and yield stress ( $F_y$  at the end of the elastic region) (Dixon, 1974). Beyond the yield stress, the apparent viscosity ( $\eta_{be}$  for example) may be calculated as the fat moves outwards with the continued narrowing of the sample gap (Casiraghi *et al.*, 1985). As in all rheological tests, experimental artefacts can complicate the analysis. Sample preparation is especially important in compression testing (Dixon, 1974). In excessively long samples, bowing occurs, while in excessively short samples, end effects can dominate (Casiraghi *et al.*, 1985).



**Figure 7.9.** Load-deformation curve for a sample of milk fat crystallized at 5°C for 24 hours.  $S$ ,  $F_y$  and  $\eta_{be}$  refer to the initial stiffness, yield stress and apparent viscosity beyond the yield stress, respectively.

### 7.3.2.1.3. *Secility Based Testing*

Secility testing can also be used to characterize plastic fats (Dolby, 1941a,b; Mohr and Wellm, 1948; deMan and Wood, 1958a; Dixon and Williams, 1977). In these tests, a stretched steel wire is forced through a sample. The load required to cut the sample correlates with firmness (Dolby, 1941a). Alternatively, the wire can be driven through the sample at a constant speed and the counteracting force measured (Hayakawa *et al.*, 1986). Again, experimental artefacts are introduced during testing (Dolby, 1941a; Luyten *et al.*, 1991; Kamyab *et al.*, 1998). The yield value and pseudoviscosity of plastic fats have been determined based on secility testing (Dolby, 1941b; Mulder and Walstra, 1974; Dixon and Williams, 1977).

### 7.3.2.1.4. *Extrusion-based Testing*

The texture of butter is sometimes evaluated by extrusion testing, although the approach is less common than those discussed previously. The justification for this type of test is that extrusion may mimic the action of spreading by a consumer. Both extrusion and spreading require rapid deformation of the material with subsequent flow (Prentice, 1972). Extruders have been designed (Prentice, 1954; Vasic and deMan, 1967) in which a sample of fat is extruded through an orifice at a constant speed. The force required to sustain the motion (i.e., the thrust) is measured. This is comprised of both the force necessary actually to force the sample through the orifice and the force necessary to overcome the friction along the walls of the extruder (Prentice, 1972). At the moment the extruder is nearly empty, the frictional component should be negligible and all the thrust related to the act of extrusion alone. By relating extrusion and sensory tests, extrusion thrust has been inversely correlated with spreadability. The extrusion friction may be related to the stickiness of the sample (Kulkarni and Rama Murthy, 1987).

### 7.3.2.1.5. *Correlation of Instrumental Methods With Sensory Testing*

The instrumental methods described above are rapid and inexpensive ways of monitoring the rheology of fats. Having said that, such tests are often criticized for not measuring fundamental properties of materials (Shukla *et al.*, 1994; Shellhammer *et al.*, 1997) and for oversimplifying complex rheological behavior (Mortensen and Danmark, 1982b). Regardless, they are powerful tools when their results correlate with some real indicator of consistency as determined by sensory panels (Dixon, 1974;

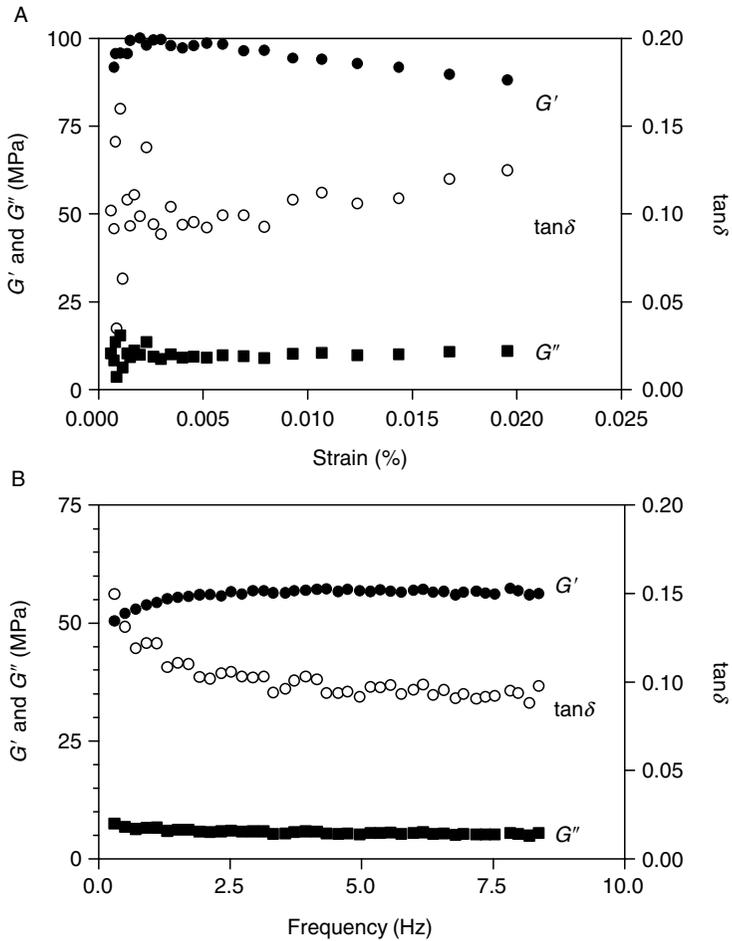
Kawanari *et al.*, 1981). Some researchers have argued that since mastication involves breaking down structure, large deformation tests are useful in understanding textural properties (Borwankar, 1992).

Several groups have reported strong correlations between large deformation tests and sensory panel evaluations (Mohr and Wellm, 1948; Kapsalis *et al.*, 1960; Haighton, 1969; Dixon and Williams, 1977; Dixon and Parekh, 1979; Mortensen and Danmark, 1982b; Davey and Jones, 1985; Pompei *et al.*, 1988; Rohm and Ulberth, 1989; Rohm, 1990; Rousseau and Marangoni, 1999). The correlation between spreadability and cone penetrometry results was shown in Figure 7.7. Although very valuable, sensory studies tend to be complicated, expensive and time consuming (Pokorny *et al.*, 1984; Kamyab *et al.*, 1998).

#### 7.3.2.2. Small-deformation Testing of Milk Lipids

Butter and milk fat exhibit viscoelastic behavior at small stresses (Chwiej, 1969; Pijanowski *et al.*, 1969; Shama and Sherman, 1970; Sherman 1976; Shukla and Rizvi, 1995). To probe this behavior, a very small stress or deformation is applied to a sample and the relationships between stress, strain and time are monitored. Viscoelastic testing is performed in the linear viscoelastic region (LVR) where a linear relationship between stress and strain exists and where the sample remains intact. Depending on the material, this region lies at a strain of less than 1.0% (Mulder and Walstra, 1974) or even less than 0.1% (Rohm and Weidinger, 1993). Figure 7.10 shows the small deformation test results for milk fat at 5°C.

Compared to large-deformation tests, small-deformation tests are very sensitive. They provide information about the structure of a sample as opposed to information about how that material breaks down. Dynamic mechanical testing also permits both the viscous and elastic components of samples to be studied simultaneously (Ferry, 1980). The testing procedure can strongly influence the results of viscoelastic testing. For example, rheological parameters are dependent on both temperature (Rohm and Weidinger, 1993; Shukla and Rizvi, 1995) and oscillation frequency (Rohm and Weidinger, 1993; Drake *et al.*, 1994). During the testing of butter, for example, the viscous modulus decreases and the elastic modulus increases as the testing frequency is increased (Diener and Heldman, 1968; Shukla and Rizvi, 1995). The viscoelastic behavior of milk fat has been studied by several groups (Sone, 1961; Chwiej, 1969; Pijanowski *et al.*, 1969; Shama and Sherman, 1970; Elliot and Ganz, 1971; Borwankar *et al.*, 1992; Wright *et al.*, 2000).



**Figure 7.10.** Storage (●) and loss (■) moduli ( $G'$  and  $G''$ , respectively) and loss tangent ( $\tan\delta$ , ○) of milk fat at 5°C as a function of strain (%) during a torque sweep at 1 Hz (A) and as a function of frequency during a frequency sweep at  $8.0 \times 10^{-3}\%$  strain (B).

### 7.3.3. Rheology of Milk Fat

#### 7.3.3.1. Setting, Spreadability, Hardness, Work Softening and Thixotropy

Several rheological characteristics of milk fat and butter have practical significance. Setting, spreadability, hardness, work softening and thixotropy are affected by the rheology of milk fat. Setting refers to the continued increase in the firmness of newly manufactured butter. Increases in firmness

sometimes occur for months because of continued crystallization (Shama and Sherman, 1968) and crystal aggregation (Precht, 1988). The extent of setting depends on several variables, including composition, storage temperature, storage time, initial hardness, and manufacturing conditions (Prentice, 1972; deMan, 1976; Precht, 1988; Shukla and Rizvi, 1995).

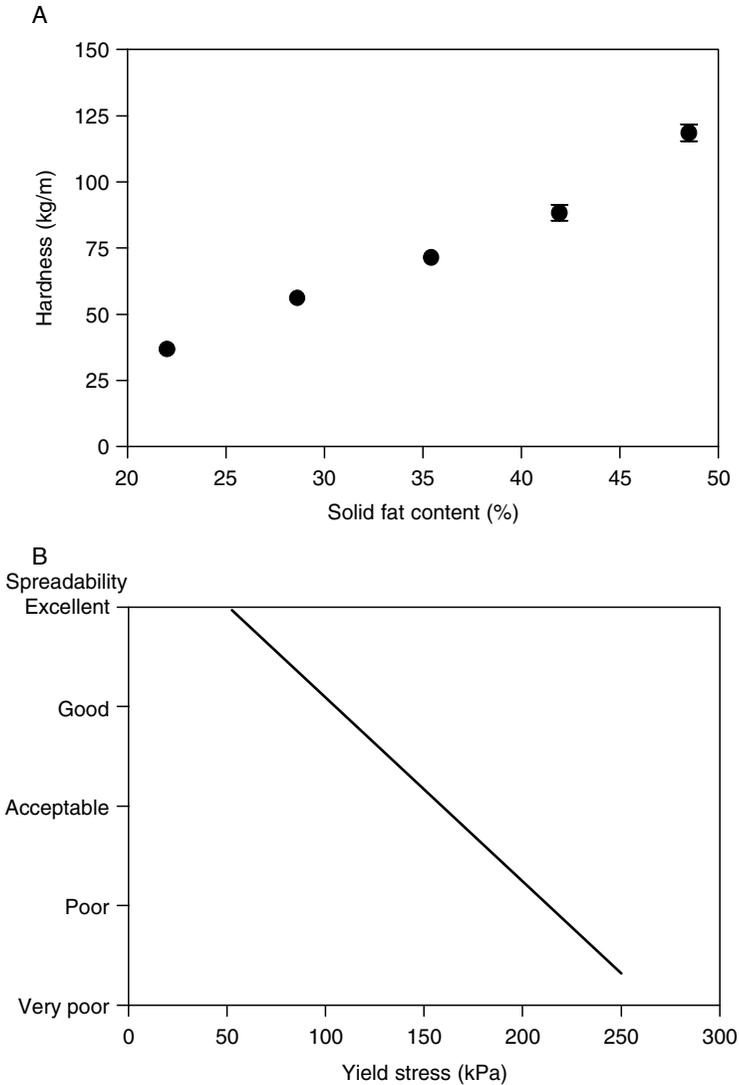
Spreadability and hardness are two very important characteristics of plastic fats (Prentice, 1972). They have a large impact on consumer acceptability. Butter is notoriously unspreadable at refrigeration temperature and is too soft or oily at room temperature (deMan and Wood 1958b; Kaylegian and Lindsay, 1992; Shukla *et al.*, 1994). The narrow temperature range for spreadability is explained by the drastic change in the SFC of milk fat between 10 and 20°C (Vasic and deMan, 1965) (see Figure 7.1) and less viscous flow at high temperatures (deMan *et al.*, 1985). Figure 7.11 shows the relationships between hardness and the SFC of milk fat (Figure 7.11A) and spreadability and the yield stress value of butter (Figure 7.11B).

To be spreadable, butter should possess an SFC between 20 and 40% (deMan, 1962) and have an apparent yield value (determined according to IDF, 1980) of 30–60 kPa (Rohm and Raaber, 1991). According to Figure 7.1, milk fat has an SFC between 20 and 40% at a temperature between 11 and 20°C. More homogeneous butter structures, as visualized by electron microscopy, have also been correlated with a firmer consistency (Precht and Buchheim, 1979, 1980).

Hardness and spreadability are inversely related. They can be investigated by sensory evaluation (deMan *et al.*, 1979; Dixon and Parekh, 1979; Mortensen and Danmark, 1982b; Pokorny *et al.*, 1984; Rohm and Ulberth, 1989) or instrumental methods as discussed previously.

Work-softening refers to the fact that when a shearing force is applied to milk fat or butter, the material softens (Shama and Sherman, 1970; Cornily and leMeste, 1985). Subsequently, thixotropic behavior in which some of the original firmness is recovered is typically observed (Vasic and deMan, 1968; deMan, 1969; Mortensen and Danmark, 1982b; deMan *et al.*, 1985). Thixotropic hardening is demonstrated by the slow increase in the elastic modulus with time after working (Shama and Sherman, 1970). Most of the instantaneous elasticity is recovered after working, although changes in viscosity are less reversible (Shama and Sherman, 1970).

The typical creep curve for a plastic fat is shown in Figure 7.8 and demonstrates the effect of working on the structure of butter. The softening that occurs in plastic fats is dependent on both the amount of force or deformation applied and also on the testing time (deMan and Beers, 1987). When a force is applied (i.e., when the sample is compressed), there is an initial elastic response (A), which can be represented by a Hookean spring (deMan *et al.*, 1985; deMan and Beers, 1987). If the yield stress is exceeded,



**Figure 7.11.** Milk fat hardness (kg/m) versus solid fat content (%) (A) and butter spreadability versus yield stress value (B) (adapted from Mortensen and Danmark, 1982).

structure breakdown occurs. Following the initial deformation, the strain continues to increase, in a time-dependent fashion. Within this region, bonds between the crystals in the fat network break and reform. This area of the curve can be represented by a series of Voigt-Kelvin units (spring and dashpot in parallel).

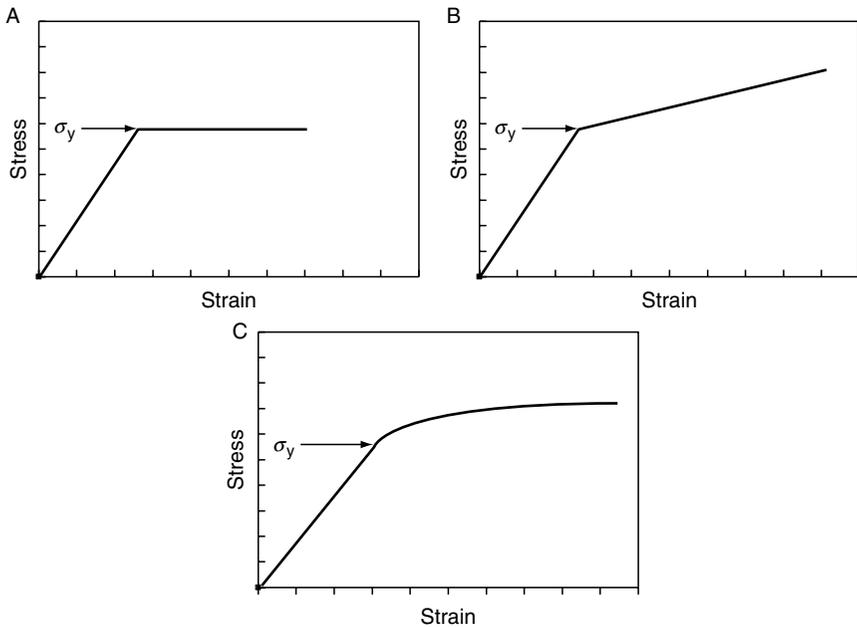
Attempts have been made to explain the response of butter to an applied force. Prentice (1972) argued that structure could be altered in three ways when butter is deformed. At a small stress, potential energy is stored because the network of the sample is distorted. This type of deformation is reversible and elastic. A larger stress causes crystals to rearrange or fracture and leads to irreversible changes in structure. Lastly, softening can occur if mechanical energy is converted into heat and leads to the melting of crystals (Sone, 1961). These changes may not be permanent because recrystallization will occur gradually but the sample may not return to its original configuration. Other groups have attempted to explain the changes in the structure of butter in the context of different bond strengths (van den Tempel, 1961; Haighton, 1963; Shama and Sherman, 1970). Primary bonds are said to be stronger and irreversible, whereas secondary bonds are relatively weak and reversible. Accordingly, primary bonds contribute more to the stiffness of a network. Thixotropic hardening may then be related to the reversible reformation of secondary bonds, which were disrupted during working (deMan and Beers, 1987). In reality, a spectrum of bond strengths is more likely than simply primary and secondary interactions (Shama and Sherman, 1970).

### 7.3.3.2. Modelling of Milk Fat Rheology

Solidified milk fat displays non-Newtonian behavior. It acts as a plastic material with a yield value (Sone, 1961; deMan and Beers, 1987). Throughout its wide melting range, milk fat, like butter, exhibits viscoelasticity, possessing both solid and liquid-like characteristics (Sone, 1961; Shama and Sherman, 1968; Jensen and Clark, 1988; Kleyn, 1992; Shukla and Rizvi, 1995). Several models to describe the complex rheological behavior of milk fat have been proposed. Figure 7.12 shows the corresponding stress-strain curves for the models discussed.

The simplest model assumes ideal elastic behavior (Figure 7.12A). At a stress below the yield stress ( $F_y$ ), the sample behaves perfectly elastically. In this region, a modulus of elasticity can be determined. At the yield stress, the sample flows. It continues to flow until the stress is lowered again to below the yield stress value. Therefore, both the elastic modulus and yield stress describe the behavior of a plastic material. They can be determined easily by compression testing. The continuous network of fat crystals in a fat bears the stress below the yield stress and therefore contributes solid or elastic properties to the material (Narine and Marangoni, 1999a).

In cases where the region of elastic deformation is very small compared the total deformation applied, the yield stress is sufficient to characterize the behavior of a material. Essentially, such a material is classified as a rigid



**Figure 7.12.** Stress-strain curves showing yield stress ( $\sigma_y$ ) for elastic-plastic behavior (A), the Elliot and Ganz (1971) model (B) and the viscous Maxwell-Bingham model of Diener and Heldman (1968) (C).

plastic material (Johnson, 1996). This simplified approach may be useful for butter (Kamel and deMan, 1975). Given the complexity of milk fat and the wide range of melting temperature of the TAGs present, we would expect that the liquid oil portion of milk fat plays a significant role in the rheology of milk fat. Consideration of the viscous elements, in addition to the elastic elements, should lead to a more accurate description of milk fat rheology. Both liquid oil and aggregated solids are responsible for the viscoelastic nature of plastic fats (Drake *et al.*, 1994).

Butter, and other unctuous materials, may be qualitatively described by a modified Bingham body (Elliott and Ganz, 1971; Elliott and Green, 1972), which consists of viscous, plastic and elastic elements in series. The stress-strain behavior for the model proposed by Elliott and Ganz (1971) is shown in Figure 7.12B. Diener and Heldman (1968) proposed a more complex model to describe how butter behaves when a low level of strain is applied. The model consists of plastic and viscous elements in parallel, coupled in series with a viscous element in parallel with a combination of a viscous and an elastic element. Figure 7.12C shows the stress-strain curve for

the proposed model. Diener and Heldman (1968) attributed the elasticity of butter to the fat globule membrane and the viscosity to the flow of the surrounding liquid fat. However, while electron microscopy has shown that fat globules can be significantly deformed without losing their structural integrity (Precht, 1988), most of the milk fat globule membrane material is lost during churning and passes into the buttermilk (Knoop and Wortmann, 1962).

### 7.3.4. Modeling Fat Crystal Networks and Relating Structure to Rheology

To model the mechanical properties of a network in terms of its structure, a good definition of the levels of structure that exist within that network is essential and a logical starting point. For fat crystal networks, the hierarchical organization of structural levels is best envisioned by considering the levels of structure that develop during crystallization. Fat crystal networks develop from initial nucleation sites, which grow into crystals as more TAGs crystallize (there may be further nucleation during growth). Growing crystals become primary particles, or microstructural elements (collection of primary crystallites or single crystals), of approximately uniform size ( $< 5 \mu\text{m}$ ). These microstructural elements then aggregate into clusters, or microstructures ( $> 100 \mu\text{m}$ ). The microstructural elements are arranged in a fractal manner in the length range bounded by the size of one microstructural element and the size of one microstructure. Figure 7.13 shows that the microstructures themselves pack in a regular, homogenous, space-filling manner. They constitute the largest structural building block of the fat crystal network. The liquid phase (oil) of the network is interspersed between the microstructural elements and microstructures.

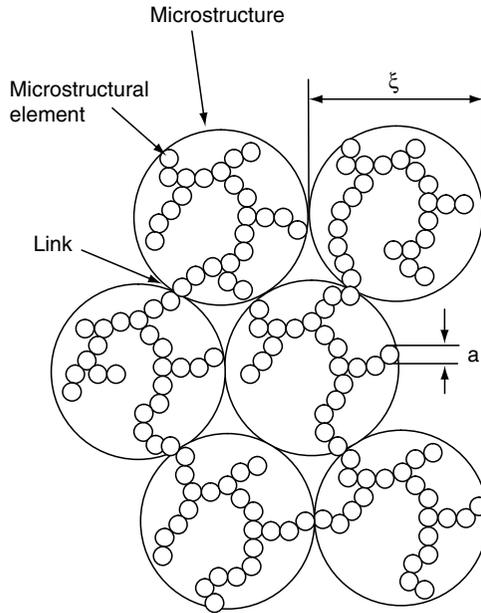
Based on this description of a fat crystal network, it makes sense that its macroscopic properties should depend significantly on the nature of the microstructures since this level of structure is closest to the macroscopic world.

Our group (Marangoni and Rousseau, 1996; Narine and Marangoni, 1999b,c; Marangoni, 2000; Narine and Marangoni, 2001) has proposed a scaling theory to relate the Young's modulus ( $E$ ) of a fat to the spatial distribution of mass within a fat crystal network, and the volume fraction of solid fat ( $\Phi = \text{SFC}/100$ ) present, namely:

$$E = \lambda \Phi^\mu \quad (6)$$

where  $\lambda$  is a pre-exponential parameter and in three-dimensional space,

$$\mu = \frac{1}{3 - D} \quad (7)$$

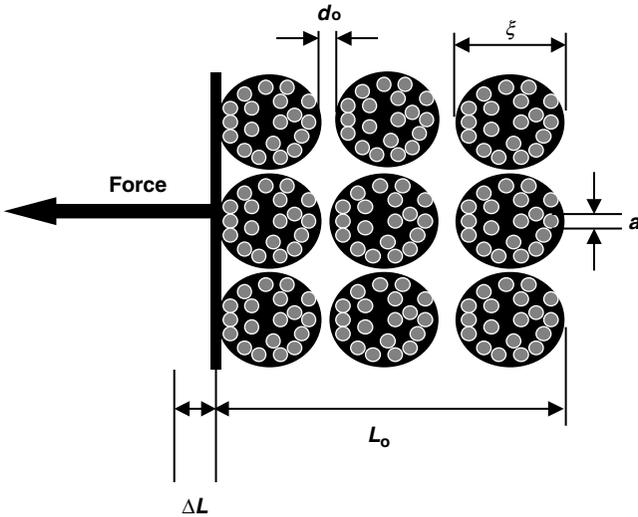


**Figure 7.13.** Schematic model of a fat crystal network showing particles of diameter  $a$  (i.e., small circles,  $< 10 \mu\text{m}$ ) arranged into clusters of diameter  $\xi$  (i.e., large circles,  $> 100 \mu\text{m}$ ) with liquid oil interspersed.

where  $D$  corresponds to the mass fractal dimension for the spatial distribution of mass within the network and is not related to the roughness of crystallite interfaces or the spatial distribution of mass within crystallites. The Young's modulus can be related to the shear elastic modulus ( $G'$ ) by assuming a Poisson's ratio of 0.5, and thus,  $E = 3G'$ .

This theory was based on the assumption that when the network is stressed, the links between the microstructures are more likely to be stressed than the microstructures themselves or the structures within them. This is in fact reminiscent of the old adage “the strength of a chain lies in its weakest link”—the weakest links here are the links between the microstructures. This theory is simply, and appropriately, called the weak-link theory. Figure 7.14 shows a schematic of a fat network under extension when the weak-link theory is applicable.

The fractal dimension of a microstructural network can be determined rheologically by diluting a fat with an oil that does not appreciably dissolve the fat under the test conditions (preferably at a low temperature and crystallized rapidly to prevent fractionation). The exact range of dilutions



**Figure 7.14.** Schematic of fat crystal network under extension when the weak-link theory is applicable ( $\xi$  is the diameter of crystal clusters,  $L_o$  is the size of the microscopic system,  $\Delta L$  is the extension due to elongational stress,  $a$  is the size of a primary particle within a cluster and  $d_o$  is the interfloc distance).

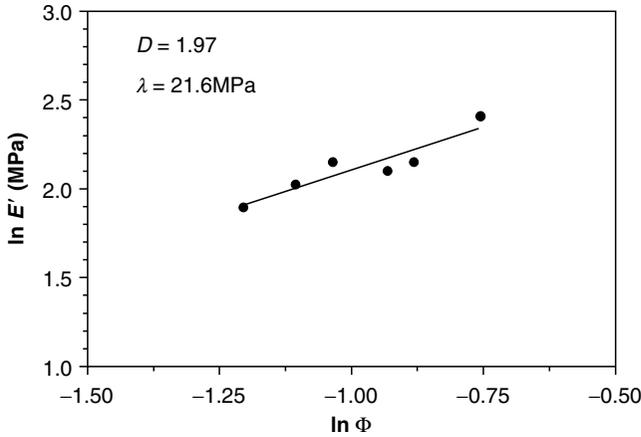
required will depend on the SFC range of interest, since different SFC ranges will yield different structures with different fractal dimensions (Awad *et al.*, 2004). Typically, dilutions in the range 70–100% (w/w) milk fat are used. By measuring the storage modulus by small-deformation dynamic rheological techniques under shear ( $G'$ ) or compression ( $E'$ ), and the SFC by pulsed NMR, it is possible to plot:

$$\log E' = \log \lambda + \mu \log \Phi \tag{8}$$

From the slope and  $y$ -intercept of such a plot it is possible to determine  $D$  and  $\lambda$ , as shown in Figure 7.15.

Our work suggests that the fractal dimension of a network is a measure of the order in the spatial distribution of the solid mass in the network as well as the degree of fill of such space. High fractal dimensions are associated with more ordered distributions and higher degrees of fill.

The fractal dimension of a crystal network is an important parameter in terms of its relation to mechanical strength. However, the values of the pre-exponential term,  $\lambda$ , (and the solid fat content) are equally important. For spherical microstructures (Narine and Marangoni, 1999c; Marangoni, 2000; Marangoni and Rogers, 2003),



**Figure 7.15.** Plot showing the relationship between natural logarithm of the dynamic compressional modulus ( $\ln E'$ ) as a function of  $\ln \Phi$  at a value for milk fat at 5°C.  $D = 1.97$  and  $\lambda = 21.6$  MPa.

$$\lambda \sim \frac{6\delta}{a\varepsilon^*} = \frac{A}{2\pi a\varepsilon^* d_o^2} \quad (9)$$

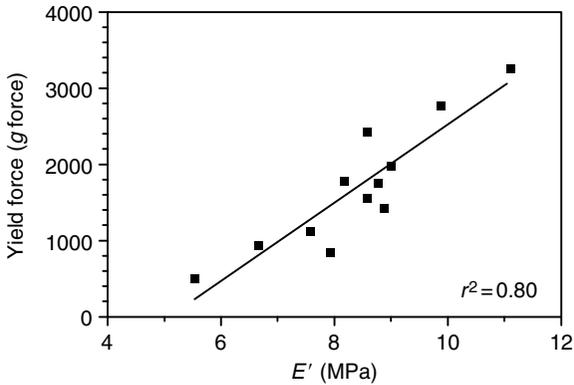
where  $\delta$  is the crystal-melt interfacial tension,  $A$  is Hamacker's constant,  $a$  is the diameter of a microstructural element,  $\varepsilon^*$  is the macroscopic strain at the limit of linearity, and  $d_o$  is the average equilibrium distance between microstructures (Figure 7.15). This model identifies key network parameters important in determining the value of  $\lambda$ . Equation 9 provides impetus for the development of investigations of relationships between TAG composition and polymorphism and the various parameters of the model in Equation 9.

In the work of Marangoni and Rogers (2003), an expression for the yield stress ( $\sigma^*$ ) of a fat was also derived assuming that  $\sigma^* = E \cdot \varepsilon^*$ , namely:

$$\sigma^* = \frac{6\delta}{a} \Phi^{\frac{1}{3-D}} \quad (10)$$

For a plastic fat, the yield stress was defined as the stress at the limit of linearity in a small deformation rheological test. Agreement between theory and experiment was found to be good.

From the above discussion, values of  $\delta$ ,  $a$  and  $D$  can be manipulated by changing processing conditions or chemical composition. This is demonstrated in the cooling rate case study at the end of this chapter. In this example, the microstructural analysis discussed is used successfully to



**Figure 7.16.** Relationship between yield stress value (g force) and compressive storage modulus ( $E'$ ) for milk fat.

describe the effects of cooling rate on the structural and rheological properties of milk fat. By defining the network characteristics responsible for the mechanical strength of a fat product, the model provides an array of indicators that can be monitored during the development of tailored fat crystal networks or as indicators for quality control purposes.

Moreover, the compression storage modulus ( $E'$ ) of milk fat was found to be directly proportional to the yield force (see Figure 7.16), in agreement with our model above. It is therefore possible to map the effects of structural changes on small deformation rheological behavior, to large deformation rheological behavior, and therefore, possibly, to texture (Rousseau and Marangoni, 1999). Work is ongoing to establish relationships between crystallization behavior, structure and macroscopic rheological properties in a variety of fat systems, including milk fat.

## 7.4. Modifying the Crystallization and Rheology of Milk Fat

Milk fat and butter can be tailored to have desired properties and functionalities. Treatments are often aimed at improving cold spreadability without compromising room temperature stability. To modify the texture and rheological properties of butter, composition and processing conditions can be manipulated

### 7.4.1. Manipulations of Butter Composition

Changes in milk fat composition can be brought about by altering the original FA and TAG composition by fractionation, hydrogenation, interesterification or blending.

#### 7.4.1.1. Changes in the Composition of Milk Fat

Several factors influence the chemical composition of milk fat and therefore the crystallization of milk fat and the consistency of butter (Bornaz *et al.*, 1993; Shi *et al.*, 2001; see Chapter 1). Fat composition varies depending on the breed of cow (Beaulieu and Palmquist, 1995), stage of lactation (Kleyn, 1992), season (Lock and Garnsworthy, 2002), and region (Collomb *et al.*, 2002). Improvements in the spreadability of winter butter and milk fat in general can be brought about through changes in the feed of the cow (Nielsen, 1971; Murphy *et al.*, 1995; Ashes *et al.*, 1997; Fearon, 2001; Hillbrick and Augustin, 2002; Gonzalez *et al.*, 2003). Feeding cows highly unsaturated oils or whole oilseeds can increase the oleic acid content of butter while simultaneously reducing the level of saturates of fatty acids (DePeters *et al.*, 1985; Mohamed *et al.*, 1988; Enjalbert *et al.*, 1997; Gulati *et al.*, 2002; Lacasse *et al.*, 2002). The result is typically a lower IV and a softer texture. Unfortunately, feeding cows polyunsaturated fatty acids can also result in an increased level of *trans* fatty acids and a decreased level of short-chain saturated fatty acids (Lin *et al.*, 1996a). *Trans* fatty acids are formed because of biohydrogenation in the rumen. To prevent hydrogenation, vegetable and fish oils can be encapsulated so that the unsaturated fatty acids can be incorporated into the milk fat (Scott *et al.*, 1970; Gulati *et al.*, 2002). When cows were fed formaldehyde-treated canola seeds, the level of oleic acid in butter was increased and the level of medium-chain saturated fatty acids reduced (Ashes *et al.*, 1992). Similarly, when high oleic sunflower oil was protected from hydrogenation by calcium, a more spreadable butter, enriched in monoenes and polyunsaturates was achieved (Lin *et al.*, 1996a,b). Milk fat composition can also be optimized for improved spreadability by supplying high enough levels of stearic acid so that desaturation in the mammary gland is maximized (Fearon and Mayne, 2000).

#### 7.4.1.2. Blending

Another way to increase the level of unsaturation in milk fat is through blending (Amer and Myhr, 1972; Ahmed *et al.*, 1979; Wilbey, 1994). When milk fat was blended with canola oil, changes in melting point and hardness index were observed (Rousseau *et al.*, 1996a,b,c). Improved spreadability at room temperature can be achieved by blending milk fat with vegetable oils. Unfortunately, this can sometimes result in instability at room temperature (Amer and Myhr, 1972). Strict standards of identity for butter also limit this type of blending in some countries, although spreads of dairy fat blended with vegetable oil are sold in many countries.

#### 7.4.1.3. The Use of Additives: Moisture, Air and Surfactants

Butter consistency can also be adjusted by manipulating its air and moisture contents (Kulkarni and Rama Murthy, 1985). When the moisture content of butter was increased from 12 to 15%, a softer texture was observed at both 5 and 15°C. Further increases in moisture content (up to 35%), however, drastically changed the rheological properties of butter (Kulkarni and Rama Murthy, 1985). The disadvantages of adding moisture to soften the texture of butter include structural stability, increased potential for microbial growth and hydrolytic rancidity, and violating standards of identity.

The air content of butter is generally between 3 and 7%, v/v. When this is reduced by working the material in a vacuum, a glossy sheen and smooth texture result. Although a harder consistency is reported, butter treated in such a way seems to show improved organoleptic plasticity, possibly because of changes in the mobility of free oil in the butter (Swartling *et al.*, 1956). Adding air or another gas, e.g., nitrogen to butter also results in significant reductions in hardness (Hayakawa *et al.*, 1986). This is the equivalent of working (Gupta and deMan, 1985) and can result in significant increases in spreadability (Vyas and Hedrick, 1963; Foley and Cooney, 1982). Several factors, including composition, manufacturing method, and the amount, type and time of gas incorporation determine the degree to which spreadability of whipped butter will be improved (Vyas and Hedrick, 1963; Precht, 1988). In some cases, butter with additional gas has been described as having a less “buttery” aroma and a more crumbly texture (Kleyn, 1992).

The addition of surfactants to milk fat may also improve butter texture (Gupta and deMan, 1985). Butter spreadability was improved in some cases, depending on the nature of the surfactant (Kapsalis *et al.*, 1963). Some surfactants resulted in a brittle and sticky product. Also, the effect of the surfactants was found to be temporary. Setting was delayed, but ultimately there was no effect of the surfactants on the SFC of butter (Kapsalis *et al.*, 1963). For this reason, surfactants have little practical significance for butter rheology (Hayakawa *et al.*, 1986).

#### 7.4.1.4. Fractionation

Milk fat fractionation has been studied by several groups (McGillivray, 1972; Makhlof *et al.*, 1987; Arul *et al.*, 1988; Deffense, 1987; Kaylegian and Lindsay, 1992; Rizvi and Bhaskar, 1995; Marangoni and Lencki, 1998; vanAken, *et al.*, 1999; Campos *et al.*, 2002a; Illingworth, 2002; Vanhoutte *et al.*, 2003). Fractionation can be used to obtain groups of TAGs with distinct chemical and physical properties and altered rheological

characteristics. For example, the spreadability of butter can be improved by fractionating milk fat and then recombining the fractions in various proportions (Kaylegian and Lindsay, 1992; Illingworth, 2002). The very high melting TAGs in milk fat seem to provide structural integrity in recombined butters, while the lower melting fractions serve to reduce hardness (Kaylegian and Lindsay, 1992). Increasing the proportion of LMF in butter increases the levels of short-chain fatty acids and oleic acid (Kulkarni and Rama Murthy, 1987). Butters enriched in the high melting TAGs have a higher SFC and viscosity at higher temperatures. This translates into improved structural stability and a reduced tendency for oiling off and moisture migration (Shukla *et al.*, 1994). The cost of fractionation can be prohibitive (Gupta and deMan, 1985) although, from a functional standpoint, fractionation is a good method for improving the physical properties of butter (Shukla *et al.*, 1994; Pal *et al.*, 2001). Milk fat fractions may also have applications in pastry-making, as chocolate bloom inhibitors (Kaylegian and Lindsay, 1992, 1994; Kaylegian, 1999), as butter flavor-rich concentrates (Bhaskar *et al.*, 1998), or to improve the rheology of reduced-fat cheese curds (Rosenberg, 2000).

#### 7.4.1.5. Interesterification

Interesterification involves the exchange of fatty acids within and between TAGs. It can be used to produce fats and oils with desired functionalities, including health benefits. Milk fat with improved cold spreadability has been produced successfully using chemical interesterification (Weihe, 1961; Mickle *et al.*, 1963; Rousseau *et al.*, 1996a,b,c; Rodrigues and Gioielli, 2003). Enzymatic interesterification can also be used to produce milk fat with altered chemical and melting properties (Kalo *et al.*, 1986a,b, 1990; Balcao and Malcata, 1998; Rousseau and Marangoni, 1999; Liew *et al.*, 2001). Unfortunately, when milk fat is interesterified, its butter flavor is, typically, reduced (Kleyn, 1992; Rousseau and Marangoni, 1999). Interesterification may be useful in incorporating healthful fatty acids, such as conjugated linoleic acid, into milk and other fats (Garcia *et al.*, 1998). This would have consequences for the physical properties of milk fat. For example, when blends of palm stearin and milk fat were transesterified, changes in polymorphic behavior were observed, with the  $\beta'$  polymorph being favored (Lai *et al.*, 2000).

#### 7.4.2. Manipulations During Processing

Processing conditions have a large impact on milk fat crystallization and texture (Campos *et al.*, 2002b; Herrera and Hartel, 2000a,b,c; van Aken and Visser, 2000; Rye *et al.*, 2005).

#### 7.4.2.1. Cooling Rate

Before butter is made, milk is first separated into cream in order to increase the fat content to approximately 40%. The cream is then pasteurized and cooled. The rate at which cooling occurs can have a very large influence on the consistency of butter. Because cooling rate influences milk fat in many ways, it can be difficult to isolate its effect on any one parameter. For example, rapid cooling influences the size of fat crystals and in turn the texture of butter (Haighton, 1976; Deffense, 1987). Cooling rate also affects the ratio of solid to liquid fat present because of mixed crystal formation (Mulder, 1953). When milk fat is cooled rapidly, a higher solid fat content and a firmer product result (Foley and Brady, 1984). Some of the increased hardness is also attributable to the formation of many small crystals during rapid cooling (deMan, 1963b; Sone *et al.*, 1966; Parkinson *et al.*, 1970).

Cooling rate dictates the number of nuclei formed during crystallization. It therefore influences the size to which crystals can grow (Bailey, 1951). Large crystals have been correlated with soft fats (Feuge and Guice, 1959). The crystal surface area also differs between small and large crystals and can influence the rheology of a fat product. For example, during rapid cooling, many small crystals form. As a result, the crystal surface area in the sample is high. This allows for more liquid fat to be absorbed than during slow cooling. In turn, the amount of liquid fat that is available to form the continuous phase in butter is reduced. The result is a firmer consistency (Boudreau and Saint-Amant, 1985).

#### 7.4.2.2. Cream Aging

Before cream is churned into butter, it is typically aged by manipulating its temperature. This is an economical and successful approach to modifying butter consistency (Precht, 1988). It has been used for nearly a century with demonstrated success. For example, butter held before churning has more free liquid oil and a softer texture than butter that is churned immediately (Dolby, 1954). Holding the cream overnight between 10°C and 12.8°C can improve the texture of winter butter (Nielsen, 1971). The Swedish or Alnarp “6-12-6” method (Alfa-Laval, 1987) is one example of a commercial procedure. Such cold-warm-cold processes result in butters with a higher level of liquid fat than samples cooled directly to lower temperatures (Szakaly and Schaffer, 1988). The success of ripening is usually attributed to the effects of liquid fat on crystal formation (Precht, 1988). During the warming process, crystals of high melting TAGs probably melt, leading to a reduction in hardness (Precht, 1988).

#### 7.4.2.3. Method of Churning

Cream is churned in order to induce a partial phase inversion and agglomeration of the partially crystalline fat and ruptured fat globule remnants. Exactly how churning is performed can have a large influence on the texture of the resulting butter (Sone *et al.*, 1966; Vasic and deMan, 1968; Black, 1975; Kawanari *et al.*, 1981). Butter was traditionally churned in batches in a large wooden or metal churn. This type of operation has largely been replaced by continuous technologies, including accelerated churning (Fritz-type), phase inversion and emulsification methods.

There are differences in the butter made by the conventional or continuous methods. Continuously-churned butter is typically harder (deMan, 1976), in part because of differences in how the fat globules are affected by the manufacturing method. When butter is batch-churned, up to 46% of the fat phase can remain in the globules. In contrast, the globule structure is completely destroyed in continuous operations such as the “Gold’n flow” process. In this case, all the fat is free (Kawanari, 1996). The degree of crystallinity and crystal morphologies also differ between churning technologies (deMan and Wood, 1958a; Sone *et al.*, 1966).

When and how any mechanical treatment is applied can also differ between the conventional and continuous approaches. In continuous operations, most of the agitation is applied before crystallization. In contrast, conventionally manufactured butter is typically agitated after most of the fat has crystallized. Agitation during crystallization can influence crystal size by increasing secondary nucleation. It can also impede crystal agglomeration and lead to more discrete crystals. This fat tends to be softer at a given solid fat content (Sherman, 1976). Milk fat crystals also tend to be larger and more irregularly shaped when batch crystallization is used (Vasic and deMan, 1968). Some of the differences in particle size are related to differences in cooling rate between the manufacturing methods. For example, cooling tends to be more rapid in the scraped-surface heat exchangers than in the traditional processes (deMan, 1963b). Crystal morphology also affects butter consistency (Sone *et al.*, 1966). Plate-shaped, disc-shaped or needle-shaped particles cause more resistance to flow than spherically-shaped crystals (deMan, 1964). In order to promote the development of desirable large crystals, butter can be seeded (Black, 1975; Joyner, 1953). While this “recycling” of crystals can lead to a softer fat, it can also adversely affect the appearance of butter (Black, 1975).

#### 7.4.2.4. Mechanical Working

Before butter is packaged, it is typically worked to achieve a desirable texture (Joyner, 1953). This step also helps to disperse the water and salt in

the continuous oil phase and promotes the release of fat crystals and oil from the fat globules (Boudreau and Saint-Amant, 1985). Reworking can be used to reduce butter hardness (MacGibbon and McLennan, 1987). Although softening occurs, some of the original firmness is regained during storage because of thixotropic setting (deMan, 1976). Beyond a certain point, working has no effect on butter hardness. With excessive working, however, butter can become sticky (Sone *et al.*, 1966; Gupta and deMan, 1985).

#### 7.4.2.4. Storage Conditions

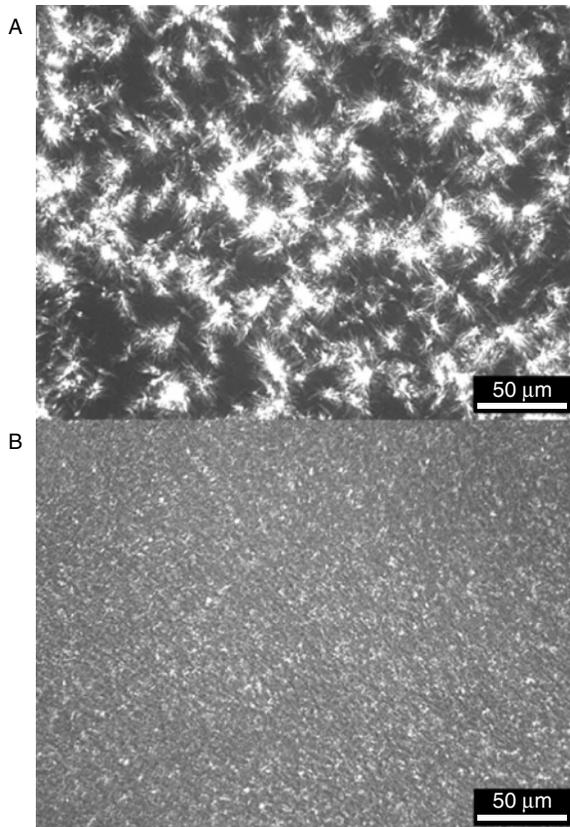
The storage time and temperature can influence the texture of butter by influencing setting (deMan, 1969; Kulkarni and Rama Murthy, 1985; Precht, 1988). The extent of setting depends on the original hardness of the butter, the composition of the fat and the manufacturing procedure (deMan and Wood, 1959; Precht, 1988). Setting tends to be more extensive in conventionally made butter than in butter that is churned continuously (deMan and Wood, 1959). Storage temperature is critical in determining the rheological properties of butter, primarily because of its influence on solid fat content. Mortensen and Danmark (1982a) observed a doubling in hardness with only a slight decrease in storage temperature.

## 7.5. Some Case Studies. Milk Fat Crystallization: Structure and Rheological Properties

Texture is an important parameter in determining the consumer's acceptance of dairy-based foods. Therefore, formulating and processing products with desirable attributes is key. Establishing relationships between milk fat composition, crystallization behavior, microstructure, and mechanical properties is therefore important. The following case studies will demonstrate how such links can be explored and the benefits of taking a holistic approach to understanding the behavior and properties of milk fat.

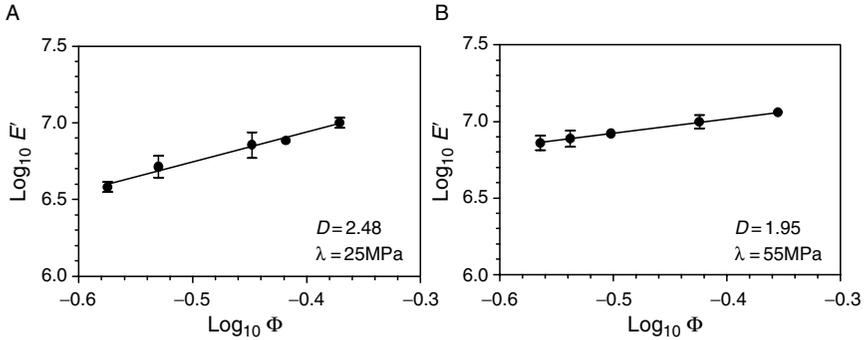
### 7.5.1. Effect of Cooling Rate on Milk Fat Crystallization and Rheology

Processing conditions, including cooling rate, influence the structure and therefore the rheology of milk fat (Campos *et al.*, 2002b). By understanding how cooling rate influences milk fat structure and, in turn, how the fat crystal network structure influences rheological properties, processors can manipulate their operations to achieve desirable outcomes. The effect of cooling rate on milk fat microstructure is shown in Figure 7.17.



**Figure 7.17.** Effect of cooling rate on the microstructure of milk fat cooled to 5°C slowly (0.1°C/min) (A) or quickly (5.0°C/min) (B).

When milk fat is cooled to 5°C at a rate of 0.1°C/min, a spherulitic microstructure is observed (Figure 7.17A). In contrast, when the same milk fat is cooled quickly from the melt to 5°C at a rate of 5°C/min, a more granular microstructure is observed (Figure 7.17B). During slow cooling, extensive crystal growth occurs. In contrast, during rapid cooling, crystallization proceeds more quickly and nucleation events predominate over crystal growth processes. The result is a large number of small microstructural features (17B). These microstructural features are distributed in a less orderly fashion than in the case of slow cooling. Figure 7.18 shows the results of the microstructural analysis, determined using a rheological approach, for slow-cooled (18A) and fast-cooled (18B) milk fat.



**Figure 7.18.** Microstructural analysis: Rheological determination of fractal dimension ( $D$ ) and exponential term ( $\lambda$ ) for milk fat (A) slowly ( $0.1^\circ\text{C}/\text{min}$ ) or (B) rapidly cooled ( $5.0^\circ\text{C}/\text{min}$ ) to  $5^\circ\text{C}$ .

The fractal dimension of the fat crystal network in milk fat decreased from 2.5 to 2.0 when the cooling rate was increased. Concomitantly, the particle-related constant,  $\lambda$ , increases. These results demonstrate how a faster cooling rate leads to a less ordered spatial distribution of mass within the microstructural network, which would result in a lower value of  $D$ , and a decrease in the average particle diameter, which would result in a higher value of  $\lambda$ , as predicted by our model. These microstructural changes were correlated with a much higher yield force value for the rapidly cooled milk fat ( $64.1 \pm 3.3\text{ N}$  versus  $33.0 \pm 3.9\text{ N}$  for the samples cooled at  $5.0^\circ\text{C}/\text{min}$  and  $0.1^\circ\text{C}/\text{min}$ , respectively).

### 7.5.2. Effect of Supplementation with Algae Meal on Milk Fat Crystallization and Rheology

The enrichment with, and incorporation of healthful lipids into milk fat is an attractive option from a nutritional standpoint. To this end, attempts have been made to increase the conjugated linoleic acid (CLA) content of cow's milk (Mansbridge and Blake, 1997; Chilliard *et al.*, 2001; Hillbrick and Augustin, 2002; Singh *et al.*, 2004). An additional benefit of increased unsaturation is altered physical properties of the milk fat, specifically, improved butter spreadability (Banks and Christie, 1990). The composition of milk fat is a major factor in determining its consistency. This was evidenced on supplementation of cows' diets with algal meal to increase the CLA content of milk fat (Singh *et al.*, 2004). The algal supplementation resulted in a threefold increase in total CLA concentration and an altered fatty acid profile overall. In particular, large decreases in  $C_{18:0}$  and  $C_{18:1cis}$ , accompanied by a large increase in  $C_{18:1trans}$ , were observed.

These compositional differences were correlated with altered crystallization and mechanical properties. The enriched milk fat crystallized much more rapidly at 19°C than the control milk. Between 20 and 27°C, nucleation induction time was reduced for the CLA-enriched milk, owing to a higher degree of supersaturation. Different polymorphic behavior was also observed for the control and enriched milk fats. At 5°C, the enriched milk fat had a greater tendency to nucleate in the  $\beta'$  polymorph while the control milk nucleated in the  $\alpha$  form. The differences in crystallization behavior translated into differences in terms of microstructure. Significantly more clustering of the crystals was observed in the enriched milk, as reflected by a lower box-counting fractal dimension over the control. In turn, the rheological properties of the milk fats were different. The enriched milk fat had a higher elastic constant (224 N/mm) than the control (100 N/mm) after 24 hours at 5°C. This occurred despite the slight decrease in SFC in the enriched milk (47.4 versus 44.4%). The study confirms the link between composition and rheological properties. Changes in crystallization behavior and microstructure are able to explain the observed trends.

### **7.5.3. Effect of Minor Components on Milk Fat Crystallization and Rheology**

Although milk fat is composed primarily of TAGs, minor lipids, including partial acylglycerols, free fatty acids, cholesterol and phospholipids are also present in small quantities. The influence of these minor components on milk fat crystallization, structure, and rheological properties has been investigated (Wright *et al.*, 2000; Wright and Marangoni, 2003). When the minor components were removed from milk fat, crystallization proceeded more rapidly. Specifically, at low degrees of supercooling (i.e., to 20°C and above) the minor components delayed crystallization either at the nucleation stage or during early crystal growth. Although crystallization was delayed, polymorphism was unaffected by the minor components and the microstructure in the absence or presence of the minor components were similar between 5 and 25°C.

The effect of the minor components was kinetic, rather than thermodynamic. Although the crystallization kinetics were altered, the structure and mechanical properties of milk fat were the same with or without the minor lipids. The samples reached the same SFC value and had a similar microstructure as observed visually and as characterized by the fractal dimension. The rheological properties of the fats were also similar. Neither the storage modulus nor the yield force was affected by removal of the minor components (Wright and Marangoni, 2003). Large changes in the

microstructure and mechanical properties of milk fat were, however, observed with changes in temperature. In this study, the composition of milk fat was correlated with changes in crystallization. However, the delay did not result in obvious or detectable changes in microstructure and mechanical properties. Instead, the results point to a kinetic effect of minor components and the possibility that such ingredients could be used as processing aids without impacting on the final quality of the product.

## 7.6. Conclusion

The crystallization behavior of milk fat is complex, owing, in large part, to its complicated composition. By manipulating composition and crystallization conditions, milk fat and dairy products with unique structures and mechanical properties can be designed. Understanding the relationships between composition, crystallization, structure, rheology and texture is a powerful tool in this regard.

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# Milk Fat: Physical, Chemical and Enzymatic Modification

M.A. Augustin and C. Versteeg

## 8.1. Introduction

Milk is an oil-in-water emulsion, in which the milk fat exists within the natural milk fat globule membrane. Comprehensive reviews are available on the structure and composition of milk fat (Christie, 1994; Jensen 2002; Chapters 1 and 2). The properties of milk fat products are influenced primarily by the fatty acid composition and the position of the fatty acids in the triacylglycerol molecule. Triacylglycerols account for  $\sim 98\%$  of the milk fat. The remaining minor lipids in milk fat comprise monoacylglycerol, diacylglycerol, free fatty acids, phospholipids, glycolipids and sterols, which although present in only small quantities, can also influence the properties of the milk fat.

The main milk fat products are butter, which is produced by churning sweet or ripened cream, and anhydrous milk fat, prepared by separation of the milk fat from melted butter or high-fat cream. Milk fat is used traditionally as an ingredient in many food applications, including bakery products, ice cream, chocolate and confectionery. Milk fat ingredients are now finding applications in ready-to-eat short shelf-life products such as dips, dressings and ready meals (Burgess, 2001).

Milk fat is valued for its pleasant flavor but its melting and rheological properties often need to be modified to make it more suitable for many food applications. The uses of milk fat can be increased by the application of various processing interventions such as fractionation, selective blending and texturization, and chemical or enzymic processes to produce speciality milk fat ingredients (Kaylegian, 1999). Most of these modification procedures

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

are concerned with changing the composition of the triacylglycerols. These treatments alter the physical and nutritional properties of the fat, enabling the tailoring of the milk fat for specific end-uses as well as expanding the range of applications. There are also other processing interventions aimed specifically at making the milk fat ingredient more attractive to the user. These are usually targeted at the minor components; one that has attracted interest over the years has been the application of processing treatments to remove cholesterol. More recently, the interest has shifted to treatments to increase the level of desirable components (e.g., conjugated linoleic acid)

There is inherently significant natural variation in the composition of milk fat, because it is affected by many factors such as cow breed, stage of lactation, season and diet of the cow (Palmquist *et al.*, 1993; Ashes *et al.*, 1997; Chapters 1 and 2). Although on-farm approaches can be used to alter the composition of milk fat to improve its nutritional composition and physical characteristics (Banks *et al.*, 1980; Banks and Christie, 1980; Hawke and Taylor, 1994; Baer, 2001; Fearon, 2001; Jensen 2002), these will not be covered here (see Chapter 2).

This chapter is focussed on the post-farm modification of milk fat by physical, chemical or enzymic means. The use and control of these processes for differentiation of milk fat to widen its application range or tailor it for specific end-applications (Mortensen, 1983; Mogensen, 1985; Boudreau and Arul, 1991; Rajah and Burgess, 1991) will be discussed. The effects of the modification processes and minor lipid components on the texture and crystallization behavior of milk fat are covered. The potential for applying modification processes to improve the nutritional quality of milk fat is also considered.

## 8.2. Physical Modification of Milk Fat

Physical modification of milk fat by fractionating milk fat or by blending milk fat or milk fat fractions with other oils and fats results in products with an altered triacylglycerol composition, but one in which the fatty acids in milk fat maintain their original position in the triacylglycerol molecules (Kaylegian, 1999).

Other methods of physical modification can involve changes in the texture of the milk fat solely by application of physical treatments to the fat without changing the triacylglycerol composition. This involves tempering or re-working the cream or butter or incorporation of air (Dixon, 1967; Mogensen, 1985). In the Alnarp cream crystallization process and variations thereof, cream is cooled rapidly to a temperature between 8 and 4°C to initiate crystallization prior to butter-making to give softer butter. After a short holding time, the cream is warmed to about 20°C at which most of the

high melting triacylglycerols crystallize, then cooled to the churning temperature for further crystallization of the middle-melting triacylglycerol. It is then held, typically, overnight before churning (Dixon, 1969). However, re-crystallization and firming occurs on storage and re-working is usually carried out. It has been shown that fast crystallization of milk fat using a scrapped-surface heat exchanger, followed by kneading of the milk fat in a working unit, reduces the apparent yield value. The kneading of the milk fat breaks the interactions between crystals but the firmness of the reworked butter increases during cool storage (van Aken and Visser, 2000). Subsequent kneading can soften the milk fat product, but the yield value increases again due to ongoing re-crystallization processes. This work shows, that although useful modifications can be made using specific crystallization and reworking techniques, the effects are moderate and the benefits are largely lost over time. Fractionation techniques offer opportunities for more enhanced and permanent modifications to physical properties.

### 8.2.1. Fractionation

Milk fat has a large number of fatty acids (>400) and a very heterogeneous triacylglycerol composition (see Chapter 1). It has a wide melting range (between about  $-40$  and  $+40^{\circ}\text{C}$ ). Milk fat lends itself to separation into a series of fractions with different chemical compositions and physical properties and this broadens its application range. Kaylegian and Lindsay (1995) presented a very comprehensive review of milk fat fractionation technology and applications of milk fat fractions in a range of products. Fractionation techniques examined include dry fractionation, solvent fractionation, supercritical fluid extraction and short-path distillation.

#### 8.2.1.1. Dry Fractionation

Dry fractionation involves melting the milk fat, controlled cooling and crystallization of molten milk fat while cooling to or at a desired temperature and separation of the crystals from the liquid phase. The process is attractive because of its simplicity, relatively low costs and ability to select between fractions based on the melting or functional properties of the fats, which is usually the reason for fractionation. It does not involve the use of solvents, detergents or other additives and furthermore, the desirable flavor notes are not lost although they are partitioned differently between the various fractions.

Dry fractionation is the most commonly used method in industry for fractionation of milk fat. After a decade of rapid growth to an installed capacity of over 800 tonnes/day in 1990 (Versteeg *et al.*, 1994), with plants in Belgium and several other European countries, growth to 2005 has been

much slower, with some plants upgraded and additional plants established in the USA, New Zealand and Spain, bringing total capacity to about 1000 tonnes/day (Deffense, personal communication).

The main commercial fractionation process for milk fat is the Tirtiaux process, followed by the De Smet process. There are also some proprietary variations of the dry fractionation process which enable the production of various milk fat fractions. The characteristics of the fractions obtained are affected by many factors, including the equipment design, the associated process, the initial temperature of the molten fat, the crystallization conditions (e.g., degree of initial supercooling), the rate of subsequent cooling and agitation after crystallization commences, the final temperature of fractionation and the method used to separate the fractions.

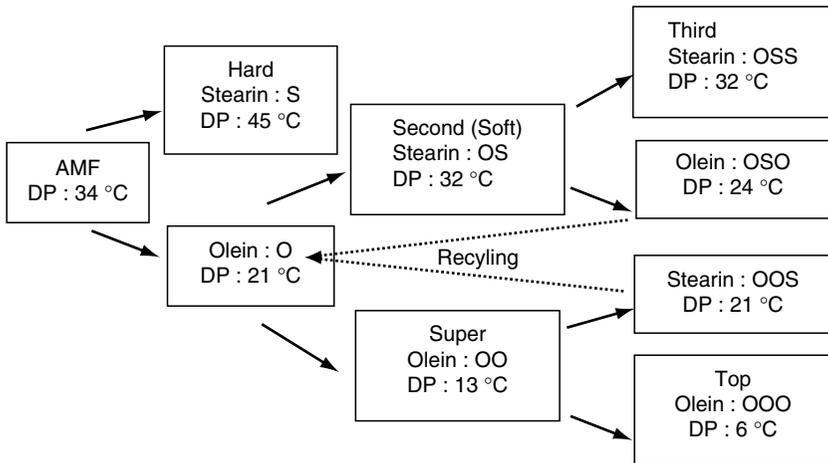
#### *8.2.1.1.1. Design of equipment for dry fractionation and process control*

Both the Tirtiaux and De Smet processes commence by melting the milk fat to 60–80°C, which is significantly above its melting point, to ensure that the highest melting triacylglycerols are molten. This is followed by rapid cooling of the melt. However, in the Tirtiaux process, the crystallization step is conducted in large insulated tanks with a relatively small heat exchange surface to volume ratio and gentle mixing. In contrast, in the De Smet process, concentric crystallizers with a relatively much larger heat exchange surface to volume ratio and much more intense agitation are used.

In the Tirtiaux process, the cooling rate is significantly reduced near the melting point and is maintained throughout the initial crystallization, during which the temperature of the oil may rise slightly, because of the heat of crystallization. This process results in the formation of  $\beta'$  crystals. After the initial crystallization, the intention of the process is to grow the existing fat crystals, but not to create new ones. Therefore slow cooling and gentle agitation is maintained until the final fractionation temperature is reached (typically between 28 and 18°C). Thus, very evenly-sized crystals can be obtained, which are easy to remove by filtration. Typically it takes about 16–20 h after commencement of the process before filtration takes place. In the De Smet process, the cooling is conducted in a number of pre-calculated steps to bring down the temperature within a few hours to the final set point of the fractionation temperature. Holding times of more than an hour may be used in both processes to complete the crystallization and increase the average crystal size. The heat of crystallization and the energy that drives the crystallization increases with the cube of crystal size whereas the opposing force of surface tension increases with the square of crystal size. Furthermore, smaller fat crystals are more soluble in the remaining liquid fat than larger crystals and redissolve, whilst the larger crystals grow at their

expense (Timms, 2005). Fat crystals in the De Smet process are much more heterogeneous in size than in a well-executed Tirtiaux process and are more difficult to remove by filtration. In the De Smet process, the crystals are ready for filtration in 6–8 h after commencing the process.

In a multistep fractionation, one or both of the fractions are reheated and taken through the process again, but at different temperature control points and with other process parameters. Commencing with a relatively soft milk fat, the first fractionation step can be conducted at about 18°C, followed by a second step at or below 10°C. When fractionating a harder milk fat, the crystal mass becomes too large and can no longer be filtered on a vacuum filter at 18°C; therefore, the first step must be conducted at a higher temperature of about 22°C, which can be followed by a second step at 12–14°C and a third step below 10°C. In a multistep fractionation of olein, metastable supersaturated fat solutions may be formed and more supercooling may be required to initiate crystallization, which makes it more difficult to control crystallization evenly. Seeding or induced nucleation techniques may be used (Deffense, 2000; Illingworth, 2002; Gibon and Tirtiaux, personal communication), speeding up the process significantly and improving the quality (separability) of the fractions. As milk fat is quite sensitive to oxidation, crystallization and filtration may be conducted under



**Figure 8.1.** Example of a 3-step fractionation process for milk fat, with optional recycling of some fractions. AMF = Anhydrous milk fat; DP = Dropping point of the fraction; O = Olein or soft fraction of given step; S = Stearin or hard fraction of given step (Gibon and Tirtiaux, personal communication).

an inert gas, such as nitrogen (van Aken *et al.*, 1999) or CO<sub>2</sub>. This is especially important in multistep fractionation of milk fat. An example of a multistep fractionation schedule is given in Figure 8.1.

#### 8.2.1.1.2. *Temperature and crystallization conditions*

There are many laboratory studies that examined isothermal crystallization using differential scanning calorimetry DSC (Foubert *et al.*, 2002; Vanhoutte *et al.*, 2002b). Models such as the Avrami and Gompertz models have been used to describe the isothermal crystallization process and Foubert *et al.* (2002) proposed a new model applicable to several fats, including milk fat, at a range of temperatures. These laboratory studies can give useful insights into factors affecting the process but may not be correlated directly to industrial processes because of differences in cooling rates and conditions for fractionation. There have been several studies on cooling rates, fractionation temperature, holding time, stirrer design, stirrer tip speed and other variables on crystallization and filtration behavior at scales from less than 1 L to more than 900 L (Deffense, 1987; Patience *et al.*, 1999; Herrera and Hartel, 2000; Vanhoutte *et al.*, 2002c, 2003; Dewettinck *et al.*, 2003). The studies of Patience *et al.* (1999) on two laboratory crystallizers (0.6 and 3.6 L) show the difficulties in translating data obtained from one scale to another. The design and operation of commercial-size crystallizers based on sound engineering principles has not been universally successful and some professional “art” remains.

The observations of Campos *et al.* (2002) showed that in non-isothermal crystallization, the slow reduction of temperature results in a lower crystal volume containing larger crystals and a more heterogeneous spatial distribution of the mass. This gives a softer fat compared to when milk fat is crystallized at a faster rate. In laboratory experiments using a Bohlin rheometer as a crystallizer, Breitschuh and Windhab (1998) demonstrated that compound crystals were formed during supercooling and that less compositionally differentiated fractions were produced.

When comparing the crystallization behavior of various milk fat fractions from multistep fractionation, van Aken *et al.* (1999) showed that the  $\beta'$  crystals were present in all fractions. The main difference between fractions was the lower rate of crystallization in the  $\beta'$  form in the lower melting fractions compared to the higher melting fractions. ten Grotenhuis *et al.* (1999) showed that very fast cooling rates of milk fat of greater than 2.5°C to -70°C/min, resulted in  $\gamma$  and  $\alpha$  crystals. At cooling rates between 2.5 and 1°C/m, only  $\alpha$  crystals were formed. At cooling rates slower than 1°C/min,  $\beta'$  crystals were formed with only a small amount of  $\alpha$  crystals. In isothermal crystallization experiments between -10 and 17°C, initially  $\alpha$  crystals were

formed, but over time they all transformed to the more stable  $\beta'$  form, but at a slower rate at lower temperatures (ten Grotenhuis *et al.*, 1999).

#### 8.2.1.1.3. Filtration

Commercially, separation is mostly performed by vacuum filtration on belt or drum filters or by pressure in membrane filters (Illingworth, 2002). Evenly-sized well-developed spherical crystals, such as those obtained in a well-executed Tirtiaux process, can be easily recovered using vacuum filters. For optimal filtration, the air on top of the filter may be cooled or heated to the filtration temperature to avoid melting or crystallization on the filter and clogging of the filter cake. This results in increasing entrainment and reducing filter speed. A considerable amount of oil is entrained in the filter cake, which can be as much as 60–70% for milk fat.

Membrane filters have a much better separation efficiency, and oil entrainment is reduced to 40–45% (Deffense, 2000). With softer and/or less evenly sized crystals, such as the crystals that tend to be obtained in the De Smet crystallization process, membrane filters are much preferred. However, membrane filters are being used increasingly for all crystallization systems to get better separations and less oil entrainment, especially in multistep fractionation, where crystals can become more difficult to filter. An added advantage is that membrane filtration is easier to conduct under an inert gas.

The filter cake is removed continuously by scraping from the vacuum filters or batch-wise after completion of the filtration with membrane filters. After removal in either system, the stearin fraction is melted for further handling and processing.

New centrifugal systems such as a filter or worm centrifuge and nozzle centrifuges (Deffense, 2000; Timms, 2005) are becoming more popular in dry fractionation. The separation efficiency of the filter centrifuge is comparable to the membrane filter. Further advantages of centrifuges include enclosed, hygienic and continuous operation (Deffense, 2000).

#### 8.2.1.1.4. Other factors affecting the process

In isothermal crystallization trials in a DSC and NMR, it was found that 0.05% and 0.5%, w/w, phospholipids could very significantly delay the onset of crystallization and crystal growth rate. The induction time was significantly increased at the higher concentrations of added phospholipid, an effect that has been attributed to the adsorption of the added phospholipid on the growth site of the fat crystal. In small-scale fractionation experiments, filtration rates were reduced by up to 20 times and oil inclusion increased, reducing the melting points of the stearins by up to 4°C (Vanhoutte *et al.*, 2002 a, b). These results show the importance of adequate

removal of phospholipids during the production of anhydrous milk fat for fractionation.

The process efficiency and yields are also affected by the triacylglycerol composition of the milk fat, the quality parameters such as the remaining water, and phospholipid concentrations (Vanhoutte *et al.*, 2002b). Shi *et al.* (2001) found a positive correlation between nucleation rate and the ratio of “solid and liquid-like” triacylglycerols in milk fats from different countries. Wright *et al.* (2000a) showed that the presence of diacylglycerols could delay the onset of crystallization with low degrees of supercooling. Particularly the *sn*-1,2 isomers are believed to be responsible for increasing the crystallization induction time and have a noticeable effect at 0.1% (w/w) (Wright and Marangoni, 2002b).

#### 8.2.1.1.5. *New process developments*

An interesting variation of the tank suspension crystallization, such as in the Tirtiaux and De Smet processes described previously, is solid-layer melt crystallization, as explored by Peters-Erjawetz *et al.* (1999). The hard fraction is crystallized on a rotating drum and scraped off continuously. No separate filtration step is needed to remove the hard fraction. Continuous dry fractionation processes have been the focus of much development work, some of which has been patented (Illingworth, 2002). However, none have yet been commercialized for milk fat. An alternative method of fractionation uses cross-flow filtration of melted butteroil on a hydrophobic membrane (Bornaz *et al.*, 1995a, b). This leads to partitioning of triacylglycerols on the basis of their molecular weight and degree of unsaturation. Thus, no crystallization step is required.

#### 8.2.1.1.6. *Properties of fractions*

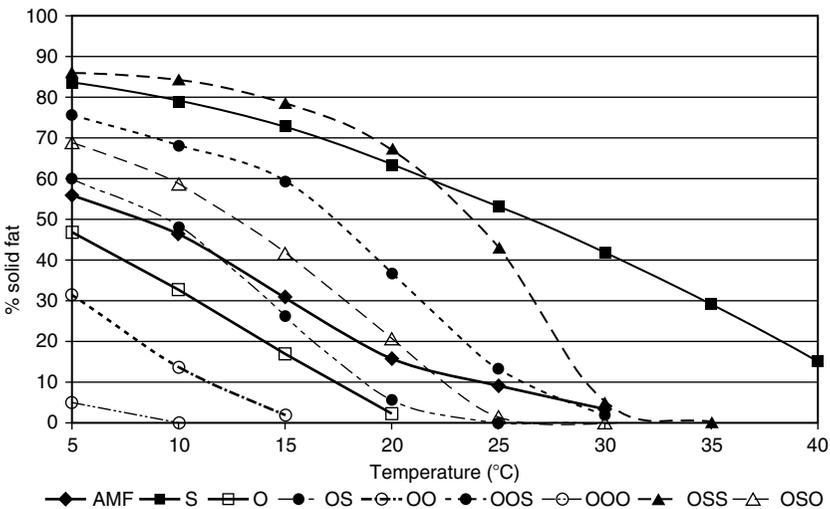
One of the attractions of dry fractionation of milk fat is that fractions with tailored melting properties can be made by choosing the fractionation conditions. Similar fractions can be produced from hard and soft milk fat, but the relative yields may differ significantly.

Typically, milk fat has a melting point of  $\sim 33^{\circ}\text{C}$ . The olein fraction from a completed crystallization and well-conducted filtration will have a melting point, softening point or Mettler Dropping point close to the fractionation temperature, regardless of the temperature or fractionation step (Deffense 1987; Versteeg 1991). If the crystallization is not completed or small crystals are squeezed through the filter cake, the olein may have a slightly higher melting point than the fractionation temperature. The solid fat content of the liquid fraction below the melting point may vary somewhat, depending on the chemical composition of original milk fat, which will

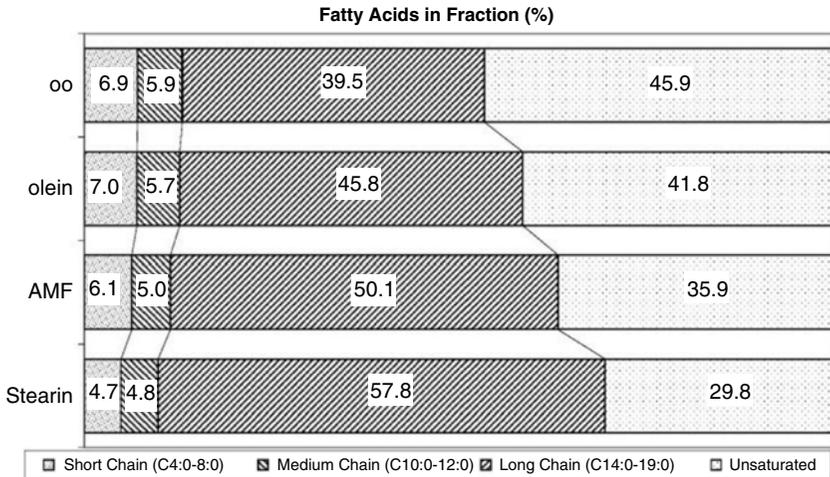
affect the properties of the fractions. Thus, in a one-step milk fat fractionation, oleins with a melting point between 18 and 30°C may be produced from the same milk fat.

The stearin separated from these oleins with vacuum filtration will typically have a melting point of 42°C (Deffense, 1987) and vary relatively little with the fractionation temperature at the first step for a given filtration system. deMan and Finoro (1980), in small scale isothermal fractionation studies, with holding time of 6 h at fractionation temperatures between 32 and 25°C, found very similar results. The melting point of the stearin is affected much more by olein inclusion than fractionation temperature and stearins obtained by membrane filtration normally have a significantly higher melting point of up to 45°C (Gibon and Tirtiaux personal communication). The fact that the stearin has a much higher melting point than the original milk fat, neatly demonstrates the phenomenon of triacylglycerol intersolubility. The high-melting triacylglycerols are dissolved in the lower-melting triacylglycerols. Once separated, the melting point of the mixture of high-melting triacylglycerols goes up by 10°C or more, from about 33 to 43°C. When decreasing the fractionation temperature, the melting point and solid fat content of both fractions are reduced.

The solid fat content and differential scanning thermograms of milk fat and its fractions obtained from a two-stage fractionation process clearly demonstrates the differentiated physical properties that may be obtained by the process (Dimick *et al.*, 1996a). Figure 8.2 gives the typical melting



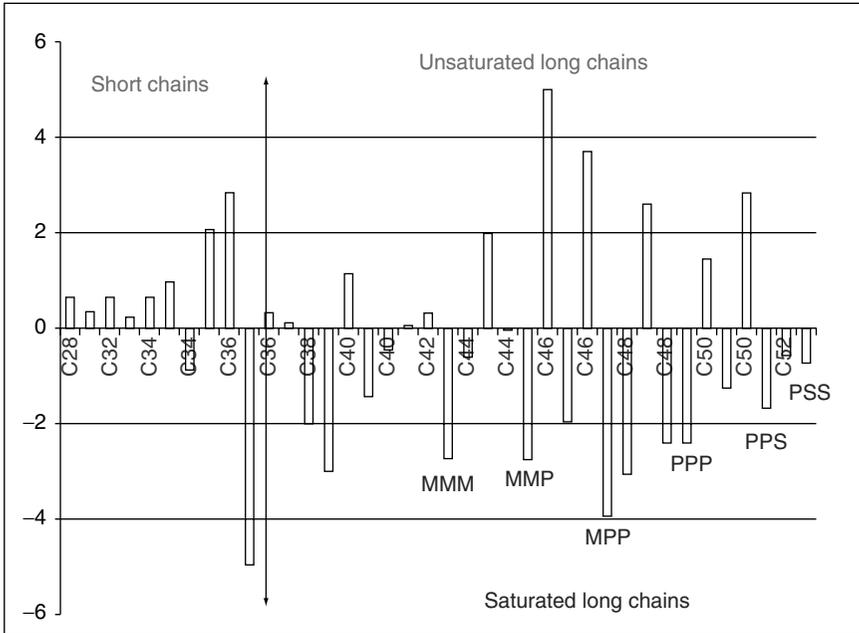
**Figure 8.2.** Solid fat content of anhydrous milk fat and fractions from a 3-step fractionation using NMR. For coding of fractions see Figure 8.1 (Gibon and Tirtiaux, personal communication).



**Figure 8.3.** Fatty acid composition of anhydrous milk fat and selected milk fat fractions grouped in short chain, medium chain and long chain saturated fatty acids and unsaturated fatty acids. For definition of fractions, see Figure 8.1. The numbers in the bars give the percentage of the group of fatty acids. Data from Deffense (1987, Table 3).

profiles of a range of milk fat fractions using NMR (Gibon and Tirtiaux personal communication). Although the individual fatty acid compositions of all fractions do not vary greatly and are not much different from the original milk fat, they are believed to be outside the seasonal variation of the individual fatty acids (deMan and Finoro, 1980; Deffense, 1987). The soft fractions are enriched in short-chain and unsaturated fatty acids and the hard fractions, in long-chain saturated fatty acids (deMan and Finoro, 1980). Figure 8.3 illustrates the change in composition of groups of fatty acids compared to the original milk fat. There are also significant differences in the triacylglycerol composition – lower molecular weight triacylglycerols with a carbon number below 42 are enriched in the oleins, and the triacylglycerols with a carbon number greater than 42 are enriched in the stearins. The difference between saturated and unsaturated triacylglycerols within each molecular weight changes between fractions. The unsaturated triacylglycerols are concentrated in the oleins and the saturated triacylglycerols are significantly reduced in the oleins (Deffense, 1987, 1993; Dimick *et al.*, 1996a). Changes in triacylglycerols in a “superolein” (melting point of 7°C) are compared with the original milk fat in Figure 8.4.

Fractionation of milk fat results in the availability of speciality milk fats that are suited to a broad range of applications, including pastries,



**Figure 8.4.** Changes in triglyceride composition of top olein (OOO) with a dropping point of 7°C compared to the original anhydrous milk fat. M = myristic acid; P = palmitic acid; S = stearic acid (Gibon and Tirtiaux, personal communication).

chocolate and confectionery. This is itself a major topic (Hartel, 1996; German and Dillard, 1998; Kaylegian, 1999; Burgess, 2001) but it has not been addressed here.

### 8.2.1.2. Solvent Fractionation

Milk fat can be separated into fractions with different melting points by crystallization in an organic solvent. Many studies have examined crystallization of milk fat in solvents such as acetone or ethanol; other solvents including hexane, pentane, ethylacetate and isopropanol have been studied also. The properties of fractions crystallized from solvents have been summarized by Kaylegian and Lindsay (1995).

The phase behavior and crystallization kinetics of the milk fat are dependent on the choice of solvent used for fractionation. Larsen and Samuelson (1979) examined the use of acetone for fractionation of milk fat. These authors suggest that the use of polar solvents has advantages over the use of non-polar solvents. The polarity of the solvent used affects the phase behavior and crystallization kinetics of milk fat. In polar solvents,

rod-shaped crystals form, whereas in non-polar solvents, the crystals are not as well defined and have a gel-like structure (Larsen and Samuelson, 1979). Wright *et al.* (2000b) have shown that crystallization of milk fat is more rapid in ethyl acetate than in hexane. The higher solubility of the triacylglycerols in non-polar solvents tends to dissolve the triacylglycerol crystals, resulting in a longer induction time. The solids content is higher when a more polar solvent is used, with the effects being more obvious at lower crystallization temperatures.

As in the case of dry fractionation, a number of fractions may be obtained using solvent fractionation, depending on the fractionation temperature. Van Aken *et al.* (1999) compared fractions of milk fat obtained by acetone fractionation and dry fractionation. Acetone fractionation yields high melting triacylglycerol fractions that are enriched in C<sub>16:0</sub> and C<sub>18:0</sub> and have reduced levels of short-chain fatty acids and unsaturated fatty acids, medium-melting fractions that are enriched in C<sub>16:0</sub> and C<sub>18:0</sub> and reduced in unsaturated fatty acids, and low-melting fractions that are reduced in C<sub>16:0</sub> and C<sub>18:0</sub> and have higher levels of short-chain fatty acids and unsaturated fatty acids. The distribution of fatty acids and triacylglycerol carbon number in fractions obtained by dry fractionation follows similar trends, except that the magnitude of the change in the fatty acid composition is not as great as that observed in fractions crystallized from acetone (van Aken *et al.*, 1999). This is because separation of crystals from a solvent is more efficient. With dry fractionation, liquid fat is included in the crystalline fraction. These differences are reflected in the yields and clear points of the fractions obtained at the same temperature when using these two fractionation techniques. However, the separation efficiency that can now be obtained by membrane filtration presses in dry fractionation, rivals that of solvent fractionation (Timms, 2005).

Despite the benefits of faster crystallization rates and better efficiency compared to dry fractionation (Schaap and van Beresteyen, 1970; Wright *et al.*, 2000b; Illingworth, 2002), crystallization of milk fat from a solvent has not been carried out on an industrial scale. Some of the hurdles to the uptake of solvent fractionation technology are the impaired flavor of the milk fat fractions, the cost of the operation, and toxicological and environmental concerns.

### 8.2.1.3. Supercritical Fluid Extraction

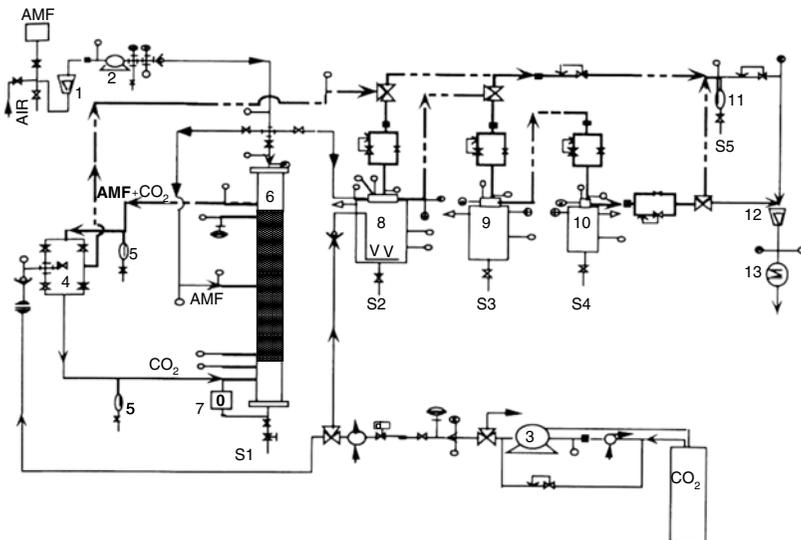
Supercritical CO<sub>2</sub> extraction may be used in batch or continuous systems to fractionate anhydrous milk fat into fractions with specific properties in order to enhance its utilization (Arul *et al.*, 1987; Bhaskar *et al.*, 1993). Supercritical fluid extraction using CO<sub>2</sub> provides an alternative to the use of

chemical solvents for the fractionation of milk fat. This fractionation technique exploits the solubility of components near the critical point of CO<sub>2</sub> and the modulation of solubility using small changes in temperature and pressure. Figure 8.5 shows a diagram of a pilot-scale continuous supercritical fluid extraction system. In supercritical fluid extraction, milk fat is fractionated into different streams selectively enriched in short-chain, medium-chain or long-chain triacylglycerols. The processing conditions for fractionation of milk fat may be varied to obtain different fractions. An advantage of supercritical CO<sub>2</sub> fractionation is that the continuous process can be used to obtain six or more fractions in one single step.

### 8.2.1.3.1. Effects of processing conditions

Kaufmann *et al.* (1982) used a pressure of 20 MPa at 80°C for fractionation of butterfat into two fractions. The short-chain fatty acids C<sub>4</sub>–C<sub>10</sub> were concentrated into the lower melting fraction, which had a melting point of 20°C compared to 37°C for the original milk fat.

Eight fractions, with a melting temperature of 9.7–38.8°C, were obtained using pressures of 10–35 MPa at 50–70°C (Arul *et al.*, 1987). The



**Figure 8.5.** Schematic diagram of pilot-scale supercritical CO<sub>2</sub> processing system. 1.– anhydrous milk fat (AMF) flow meter; 2. AMF pump; 3. CO<sub>2</sub> pump; 4. CO<sub>2</sub> loop; 5. flow loop; 6. entrainment vessels; 7. view cell; 8. separator 1; 9. separator 2; 10. separator 3; 11. separator 4; 12. CO<sub>2</sub> meter; 13. dry test meter (Reproduced with permission from Bhaskar *et al.*, 1993).

average molecular weight of the fractions increased with an increase in melting temperature, suggesting that supercritical CO<sub>2</sub> extraction leads to a significant degree of molecular weight separation.

Operating in the pressure range of 24.1–3.4 MPa and at a temperature of between 40–75°C, Bhaskar *et al.* (1993) separated the original milk fat (melting point 40°C) into 5 fractions with a melting point ranging from 42.9 to 28.4°C. Fractions with a higher melting point had a lower saponification value (i.e., longer fatty acid chain length) and a higher iodine value (i.e., a higher content of unsaturated fatty acids). In fractions with long-chain fatty acids, the effect of the higher content of unsaturated fatty acids on reducing the melting temperature of the raffinate was off-set by the higher proportions of long-chain saturated fatty acids.

#### 8.2.1.3.2. *Comparison between supercritical CO<sub>2</sub> extraction and dry fractionation*

The fractions obtained by supercritical CO<sub>2</sub> extraction are different from those by dry fractionation. There are differences in the fatty acid and triacylglycerol compositions and melting profiles (Table 8.1). As the characteristics of the fractions depend on the conditions of the processes and the number of fractions obtained, any comparison between fractions should take into account not only the type of processes employed but also the specific process conditions.

The two processes have been compared (Rizvi and Bhaskar, 1995; Bhaskar *et al.*, 1998). Differences in the carbon number of triacylglycerols were more marked between solid and liquid fractions from supercritical CO<sub>2</sub> fluid extraction than when melt fractionation was used (Table 8.1). Differences in the fatty acid and triacylglycerol compositions of fractions obtained using these two processing technologies were, as expected, reflected in differences in solid fat content and DSC profiles. The differences in the chemical and physical properties of the fractions are due to the differences in the basis of fractionation—with supercritical CO<sub>2</sub> fluid extraction, fractionation is based on molecular weight and the dielectric constant whereas in dry fractionation using melt crystallization, the basis for separation is the difference in the melting point of the various components in the milk fat.

There is a potential for the commercial application of supercritical CO<sub>2</sub> fluid extraction for fractionation of milk fat. However, the differences between the melting properties are not as pronounced as with melt crystallization, thereby limiting the application range of these fractions. Moreover, this process is more expensive than melt crystallization (Bhaskar *et al.*, 1998). Nevertheless, niche applications could be developed if fractions rich

**Table 8.1.** Physical and chemical properties of milk fat fractions obtained using fractionation based on melt crystallisation or supercritical CO<sub>2</sub> fluid fractionation<sup>a</sup>

Process Fraction	Fractionation by melt crystallization <sup>b</sup>					Supercritical CO <sub>2</sub> fluid fractionation				
	AMF45	AMF30	AMF20	AMF10	Milk fat	Super stearin	Stearin	Olein	Super olein	
<i>Fatty acid (%)</i>										
Saturated	72.6	66.06	66.35	58.59	63.94	51.41	63.06	67.02	70.66	
Unsaturated	20.22	26.29	26.29	33.34	28.58	38.96	29.48	25.99	23.13	
Ratio <sup>c</sup>	0.28	0.40	0.40	0.57	0.45	0.76	0.47	0.39	0.33	
<i>TAG (%)</i>										
C24-C34	7.11	9.75	11.44	14.47	8.08	0.20	4.89	10.38	18.97	
C36-C40	24.78	30.90	36.44	34.31	30.26	2.53	27.52	38.75	42.79	
C42-C54	56.75	50.32	44.42	42.79	53.99	90.58	58.44	43.51	34.15	
<i>SFC (%)</i> <sup>d</sup>										
0°C	78.9	60.8	56.9	30.8	61.9	80.8	65.6	58.2	52.4	

<sup>a</sup> Data from Bhaskar *et al.* (1998).

<sup>b</sup> Commercial fractions.

<sup>c</sup> Unsaturated/saturated.

<sup>d</sup> solid fat content after 24 h.

in short-chain fatty acids or a solid fraction high in unsaturated fatty acids are required (e.g., for nutritional purposes).

#### 8.2.1.4. Short-path Distillation

##### 8.2.1.4.1. *Effects of processing conditions*

In short-path distillation, molecules are volatilized into a vacuum. By this method of molecular distillation, milk fat fractions with unique chemical and physical properties may be obtained. The fatty acid composition of the fractions is dependent on the distillation temperature and there is a high degree of molecular weight separation between the fractions (Boudreau and Arul, 1991). The solid fat content of distillates is lower than that of the original milk fat whereas that of the retentate, which is enriched in high-molecular weight triacylglycerols, is higher (Arul *et al.*, 1988a; Campos *et al.*, 2003). Increasing the distillation temperature from 125 to 250°C increases the yield of the distillate from 0.3 to 42.7% and increases the hardness of the retentate (Campos *et al.*, 2003).

##### 8.2.1.4.2. *Comparison of short-path distillation and other fractionation processes*

Rizvi and Bhaskar (1995) compared the properties of milk fat fractions obtained by short-path distillation (vacuum distillation in three steps between 245–265°C between 200 and 100 µmHg; Arul *et al.*, 1988a) with supercritical CO<sub>2</sub> fluid extraction (24.1–3.4 MPa; 40–75°C; Bhaskar *et al.*, 1993) and melt crystallization (Makhlouf *et al.*, 1987). Trends for fatty acid and triacylglycerol compositions were similar for the three techniques in solid, intermediate melting and liquid fractions with respect to carbon chain length but the proportions obtained varied. The concentrations of short-chain and medium-chain fatty acids (C<sub>4</sub>–C<sub>12</sub>) in liquid fractions were highest when short-path distillation was used compared to liquid fractions from melt crystallization or supercritical CO<sub>2</sub> fluid extraction. Unsaturated fatty acids increased in the liquid fraction when melt crystallization was used but decreased with the other two fractionation techniques. The ratio of unsaturated to saturated fatty acids for liquid fractions was lowest in the liquid fraction from short-path distillation. Comparing the solid fraction, the ratio of unsaturated to saturated fatty acids in the fraction from short path distillation was intermediate between those obtained using melt crystallization and supercritical CO<sub>2</sub> fluid extraction (Rizvi and Bhaskar, 1995). Although the fractions from short-path distillation have distinctive properties compared to those obtained using other fractionation techniques, it has not been used by industry. Short-path distillation has the disadvantage of

decomposing and polymerizing unsaturated fatty acids and has high thermal energy demands (Boudreau and Arul, 1991).

## 8.2.2. Physical Blends of Milk Fat with Other Fats and Oils

Increasingly, milk fat is blended with other edible fats or oils to obtain products with desired functional properties in order to increase the application range of milk fat products, whilst generally reducing ingredient costs.

### 8.2.2.1. Softening of Milk Fat

Milk fat fractions are blended with milk fat in cream or other fractions to give spreadable butters (Deffense, 1987, 1993). Milk fat can also be softened by blending with liquid vegetable oils such as canola oil (Rousseau *et al.*, 1996a). The addition of about 20% vegetable oil to cream before churning to make a spreadable butter-based blend was introduced in Sweden as Bregott as early as 1969 (Anon, 1969). In countries where the legislation permits, these types of blends, table spreads consisting of butter/vegetable oil blends with 20–40% vegetable oil of the total fat, have become very popular. In Australia in 2003/2004, more than 53% of the milk fat-based table spreads were sold as “dairy blends,” with traditional butter accounting for the other 47%. The dairy blend category was the only table spread growing in volume, largely at the expense of margarines (Dairy Australia, 2004).

### 8.2.2.2. Hardening of Milk Fat

Milk fat may be made harder by blending with high melting triacylglycerols or fats. Blending of milk fat with tripalmitin has been suggested to make it more suitable for applications as moisture barriers in edible films and coatings. Blending milk fat with 10–20% of tripalmitin is an alternative to the use of hard milk fat fractions to increase the mechanical properties of coatings (Fairley *et al.*, 1994). Blending milk fat with beef tallow and beef tallow fractions increases the hardness of the fat and increases its physical functionality in shortening applications (Timms, 1979).

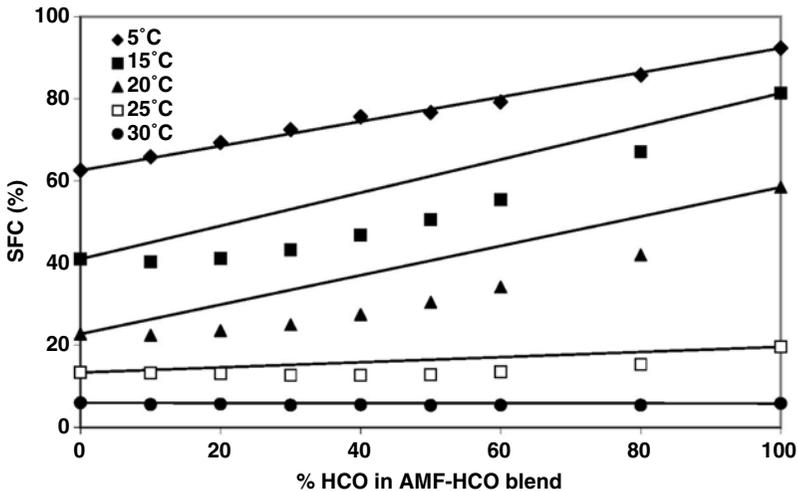
### 8.2.2.3. Effects of Blending on Crystallization Behavior

Research on blending milk fat and its fractions with cocoa butter to reduce costs and/or to improve stability against fat bloom in chocolates has had mixed results. Milk fat hard fractions have been reported to inhibit fat bloom formation in both milk and dark chocolates (Versteeg *et al.*, 1994; Dimick *et al.*, 1996b; Bricknell and Hartel, 1998) but to accelerate fat bloom in compound coatings based on palm kernel oil (Ransom-Painter *et al.*, 1997). Reddy *et al.* (1996) showed the importance of modifying the

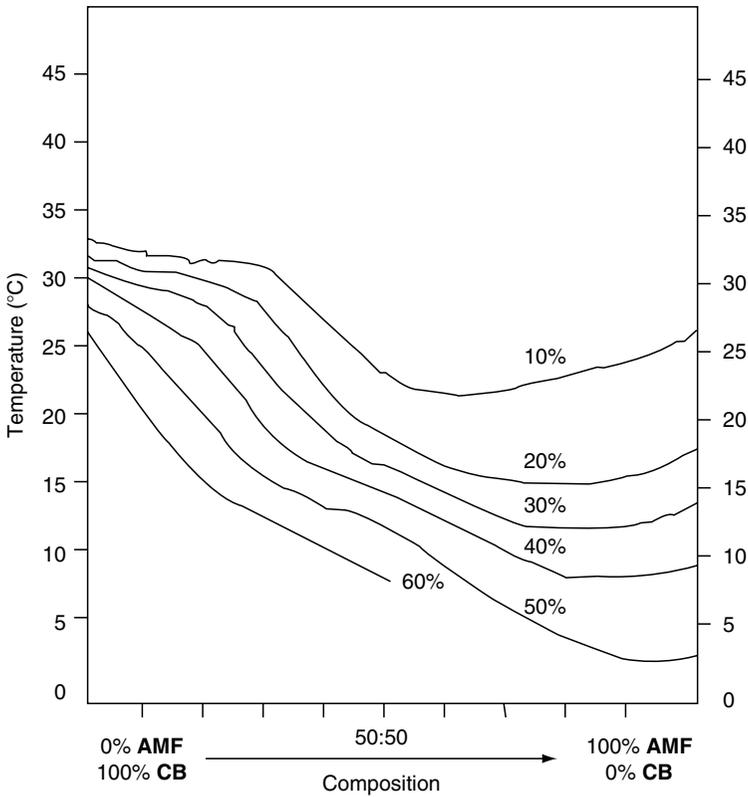
tempering procedures to take into account the different crystallization behavior of the blends, in order to obtain good texture and stability.

While blending fats, often the melting properties can be different from the arithmetically calculated average based on the composition of the blend. Intersolubility can occur (e.g., the harder fat dissolves in the softer fat, thus significantly lowering the melting point) resulting in eutectic interactions. An example of this is given in Figure 8.6 for blends of milk fat with hydrogenated coconut oils (Shen *et al.*, 2001).

In isosolids diagrams, which can be constructed for mixtures of two or three fats, each line shows at which temperature a selected solids percentage is obtained for all blends in the studied range. When the lines are straight, the fats are believed to be compatible. The presence of eutectic and compound interactions has been observed in blends of milk fat and beef tallow where there are deviations from linearity of the isosolid lines above the 40% solid-fat line (Timms, 1979) and also in blends with cocoa fat (Hartel, 1996; Figure 8.7) and palm oil stearins (Nor Hayati *et al.*, 2000). After *sn*-1, 3-interesterification of the blends, the eutectic effects were reduced. Hartel (1996) concluded that, based on the isosolid diagrams, milk fat fractions are fully compatible with palm kernel oil, yet they caused significant fat bloom and softening in confectionary coatings. It was demonstrated that



**Figure 8.6.** Solid fat content in anhydrous milk fat (AMF) and hydrogenated coconut oil (HCO) and their blends. Symbol – measured data; Straight line – weighted average; (Reproduced with permission from Shen *et al.*, 2001).



**Figure 8.7.** Isosolid diagram of cocoa butter (CB) (Malaysian/Brazilian blend) with anhydrous milk fat (AMF) (Reproduced with permission from Hartel, 1996).

blends of 50–70% anhydrous milk fat with soft palm stearin and hard palm stearin could give similar melting characteristics to a milk fat hard fraction (Nor Hayati *et al.*, 2002).

**8.2.3. Modification of Milk Fat Properties by Addition of Minor Lipids**

The minor lipid components in fats, such as partial glycerides, free fatty acids and phospholipids, influence crystallization behavior and the rheological properties of fat. Early workers demonstrated that the spreadability of butter could be improved by adding of 1% milk fat monoacylglycerols (Gerson and Escher, 1966). Several additives, including Tweens, Spans, lecithin, buttermilk solids and skim milk solids were effective in improving the spreadability of butter. However, the beneficial effects were

not evident after prolonged storage as the emulsifiers delayed, but did not prevent, the hardening of butter (Kapsalis *et al.*, 1963). The whipping properties of cream were superior when phospholipids were added to cream (Thome and Eriksson, 1973).

Minor lipids can have a significant influence on the kinetics of crystallization of milk fat. The effects on the kinetics of crystallization processes should be separated from their influence on the final properties of the milk fat. The effects of minor lipids on the modification of milk fat properties are complex and are dependent on the type, chemical nature, concentration and structure of the minor lipids (Wright and Marangoni, 2002a; Vanhoutte *et al.*, 2002a).

#### 8.2.3.1. Effects of Minor Lipids on Milk Fat

The crystallization behavior of milk fat (which contains minor lipids) and a pure triacylglycerol fraction of milk fat were compared by Herrera *et al.* (1999). The results suggested that minor lipids delay nucleation but promote crystal growth. Other workers who examined the effects of added phospholipids on palm oil, suggested that some phospholipids delayed nucleation while others had more significant effects on the rate of growth of fat crystals (Smith, 2000).

Wright *et al.* (2000a) examined the effects of adding diacylglycerols at 0.1%, w/w, to milk fat triacylglycerols. The thermodynamic equilibrium was not affected by adding diacylglycerol at this level. This was reflected in the unchanged melting point and final solid fat content and similar milk fat microstructure of systems with or without added diacylglycerol (Wright *et al.*, 2000a; Wright and Marangoni, 2002b).

However, the addition of 0.1%, w/w, diacylglycerols, isolated from milk fat, influenced the kinetics of crystallisation of milk fat (Wright *et al.*, 2000a). Between 5 and 15°C, the induction time for crystallization, measured by the solid fat content for crystallisation, was not affected. At a higher temperature (20–27.5°C), the added diacylglycerols delayed the onset of crystallisation. However, consistent results were not obtained. Differences were observed with different batches of milk fat; in one, the addition of milk fat diacylglycerols had a significant effect on crystallisation of milk fat triacylglycerols, but in another there was only a small effect. It was suggested that the differences in the positional distribution of fatty acids between the diacylglycerols could have contributed to their differing effects on the rates of crystallization (Wright and Marangoni, 2002a).

Studies on the addition of standard diacylglycerols demonstrated that their racemic purity had a significant influence on crystallisation behavior of milk fat triacylglycerols. Differences in the observed effects on crystallization

behavior may be attributed to the extent of the molecular interactions between the diacylglycerols and triacylglycerols (Wright and Marangoni, 2002b). Research on the effects of diacylglycerols on another oil, palm oil, also showed that there could be differing effects on the inhibition of nucleation and crystallisation depending on the nature of the diacylglycerols added and their solubility (Siew and Ng, 1999).

#### 8.2.3.2. Effects of Minor Lipids on Milkfat Blends

Tietz and Hartel (2000) studied the effects of removing or adding minor components naturally present in milk fat on the crystallization of milk fat–cocoa butter blends. They suggested that at low concentrations, minor lipids act as sites for nucleation and promote the rate of crystallisation and at higher concentrations inhibit crystallisation. They concluded that the presence of minor lipids, at the concentrations naturally occurring in milk fat, were sufficient to affect crystallisation rates, chocolate microstructure and fat bloom formation in chocolate.

### 8.3. Chemical Modification of Milk Fat

Milk fat may be chemically modified to obtain products with altered functionality. In contrast to physical modification of milk fat where the position and nature of the fatty acid chains of the triacylglycerols are maintained, the use of chemical processes results in modification of the composition of the fatty acid chains or their positions in the triacylglycerol molecule.

#### 8.3.1. Hydrogenation

Hydrogenation involves the addition of hydrogen atoms to the double bonds in the fatty acid chain. The process is carried out at a high temperature in the presence of a catalyst. Hydrogenation reduces the degree of unsaturation of fat and increases its hardness. It is used on an industrial scale in the vegetable oil industry.

Research has shown that a low level of hydrogenation of milk fat improved its oxidative stability (Mukherjea *et al.*, 1966), an effect that is to be expected due to the decrease in unsaturation. Hydrogenation of milk fat to different extents enables the production of fats with higher melting points and blends of these can be made to suit specific end-uses (Huyghebaert *et al.*, 1986). It is unlikely that hydrogenation will be used in the industry because making milk fat more saturated is not desirable from a nutritional viewpoint. Hydrogenation increases the level of *trans* fatty acids, which are of concern from a nutritional point of view (Timms, 2005).

### 8.3.2. Chemical Interesterification

Interesterification involves an exchange of acyl groups within and between triacylglycerol molecules. This re-distribution of the fatty acids results in modification of the physical properties and nutritional properties of the fat (Frede, 1991). The traditional process of interesterification involves the use of chemicals.

Chemical interesterification randomizes the fatty acid distribution in the triacylglycerol. The extent of modification of the fat depends on the composition of the starting fat and whether a single or a blend of fats is used and the conditions of the chemical interesterification process (Mickle *et al.*, 1963; Huyghebaert *et al.*, 1986; Rousseau and Marangoni, 2002).

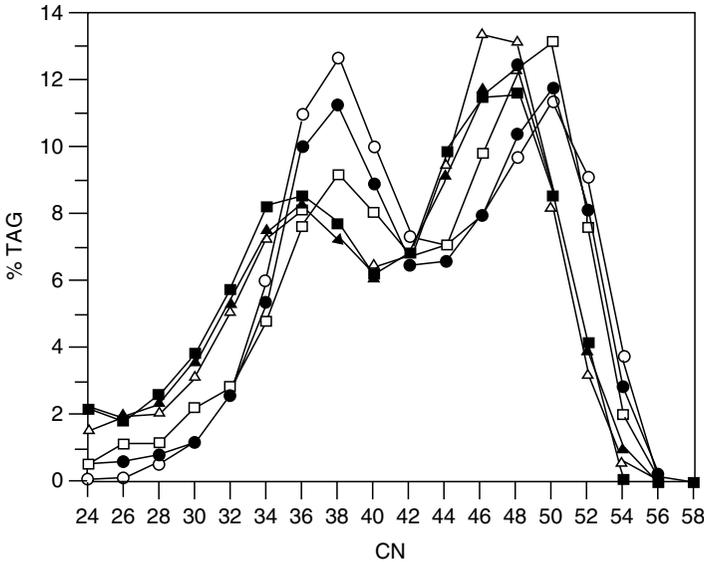
#### 8.3.2.1. Chemical Interesterification of Milk Fat

Early work showed that interesterification of milk fat resulted in an increase in the melting point of milk fat and the concentration of high melting triacylglycerols (de Man, 1961). The effect on the melting point of milk fat was greater in the case of directed interesterification compared to random interesterification. The use of solvents in the interesterification process also enhances its effects on the melting point of the modified milk fat (Weihe, 1961).

A reduced level of low molecular weight monounsaturated triacylglycerols ( $C_{36}$  and  $C_{38}$ ) and increased levels of trisaturated triacylglycerols ( $C_{44}$ – $C_{50}$ ) was obtained on undirected chemical interesterification of milk fat with sodium methoxide (0.5%) as catalyst, resulting in a wider temperature range for crystallization compared to native milk fat (Parviainen *et al.*, 1986).

Intesterification of milk fat at 90°C with sodium methylate as catalyst (0.1–0.3%) for 1 hour significantly altered the ratio of low : high molecular weight triacylglycerols. Huyghebaert *et al.* (1986) used the ratio of  $C_{38}/C_{50}$  as an indicator of the change on interesterification of milk fat. Intesterification reduced the ratio of  $C_{38}/C_{50}$ , demonstrating the increased level of the higher molecular weight triacylglycerol in the interesterified milk fat. Changes in the solid fat content of the milk fat were also obtained after interesterification.

Rousseau *et al.* (1996a) showed that chemical interesterification of milk fat (using 0.5% methoxide at 78–82°C for between 15–120 min) changed the triacylglycerol composition of the milk fat (Figure 8.8). It caused a decrease in the lower melting triacylglycerols and an increase in dropping point. Changes in the solid fat content were obtained, with the interesterified milk fat having lower % solids below 15°C. The trend of reduced solid fat content at low temperature (5–15°C) and increased solid



**Figure 8.8.** Effect of chemical interesterification on the relative proportion (w/w) of milk fat triacylglycerols as a function of carbon number (CN). TAG = triacylglycerol. Noninteresterified milk fat (○-○), interesterified milk fat-15 min (●-●), 30 min (□-□), 60 min (■-■), 90 min (△-△), and 120 min (▲-▲). (Reproduced with permission from Rousseau *et al.*, 1996a).

fat content as the temperature is raised to 35°C with chemically interesterified milk fat (Rousseau *et al.*, 1996a) was similar to that observed previously (Huyghebaert *et al.*, 1986). The higher dropping point of the chemically interesterified fat (37.0°C) compared to the unmodified milk fat (34.4°C) was considered to be due to a more structured and denser crystal network of the interesterified milk fat (Rousseau *et al.*, 1996b).

When interesterification of milk fat was carried out at 100°C with 0.2% sodium, there was an increase in middle-melting point triacylglycerols but only small effects on the melting properties of milk fat (Timms and Parekh, 1980). These authors concluded that although the interesterified milk fat was more compatible with cocoa butter than unmodified milk fat, the effects were not sufficient to warrant the use of interesterification (Timms and Parekh, 1980).

### 8.3.2.2. Chemical Interesterification of Fat Blends Containing Milkfat

The use of a blend of milk fat and other fats as the starting material enables a wider range of physical properties to be achieved than when milk

fat alone is used for interesterification. The properties of physical blends of milk fat with another fat are significantly different from that of a chemically interesterified blend.

#### 8.3.2.2.1. *Milkfat–beef tallow blends*

From the 50% solid fat line in the isosolid diagram of physical blends of milk fat and beef tallow, it was evident that there were eutectic and compound interactions but these were absent in interesterified blends with a more complex triacylglycerol composition (Timms, 1979).

#### 8.3.2.2.2. *Milkfat–corn oil blends*

A comparison of physical mixtures of milk fat–corn oil and of blends that were interesterified (0.5% sodium methoxide, 65–70°C under reduced pressure) showed that interesterification increased the softening point of the blends (Rodrigues and Gioielli, 2003). There were only small changes in the total disaturated-monounsaturated and monounsaturated-diunsaturated triacylglycerols but there were marked differences in the symmetrical and asymmetrical contents of the triacylglycerols

#### 8.3.2.2.3. *Milkfat–canola oil blends*

Rousseau *et al.* (1996a, b) examined the properties of physical blends and those of blends obtained on chemical esterification of milk fat–canola oil blends (using 0.5% methoxide, 78–82°C for 15–120 min). For milk fat–canola oil blends containing  $\geq 20\%$ , w/w, canola oil, chemical interesterification reduced the solid fat content of all blends (Rousseau *et al.*, 1996a). For non-interesterified and interesterified blends, hardness decreased with increasing content of canola oil.

At the same milk fat: canola oil ratio, interesterified blends were softer than the non-interesterified blends, an observation that was attributed to the combined effects of the dilution of the milk fat by the softer canola oil and the incorporation of the unsaturated fatty acids of the canola oil into the triacylglycerol molecules of the milk fat (Rousseau *et al.*, 1996b).

## 8.4. Enzymic Modification of Milk Fat

Although the processes of hydrogenation and chemical interesterification that are commonly used in the wider oils and fats industry may be applied to milk fat, there are a number of factors that prevent these being an attractive option for milk fat modification. Apart from the fact that ingredient manufacturers are seeking alternatives to the use of chemicals, milk fat is

expensive compared to most vegetable oils and fats and the delicate desirable butter flavor for which milk fat is valued, is lost under the conditions normally used for chemical processes. An alternative to the use of chemicals is to use enzymes because gentler processing conditions may be used. The most common enzyme used is lipase (glycerol ester hydrolase, EC 3.1.1.3) that hydrolyses triacylglycerols, diacylglycerols and monoacylglycerols and under certain circumstances may also catalyse the re-incorporation of free fatty acids onto the glycerol backbone.

### 8.4.1. Enzymic Interesterification

#### 8.4.1.1. Types of Lipases and Their Action

Lipases may be used to lipolyse milk fat to produce dairy flavor enhancers or for interesterification of milk fat systems to produce milk fat with improved nutritional or physical properties. Lipases may be used with or without an organic solvent in the system (de Greyt and Huyghebaert, 1995; Rousseau and Marangoni, 2002).

Lipases can be classified into groups that reflect their specificity. The common lipases include non-specific lipases that do not discriminate between the position or the type of the fatty acid on the triacylglycerol (e.g., lipase from *Candida cylindracea*) and 1,3-specific lipases that act only at the *sn*-1 and *sn*-3 positions of the triacylglycerol (e.g., lipases from *Aspergillus niger* and *Rhizopus* species). In addition, some lipases are specific for a specific fatty acid type (e.g., lipase from *Geotrichum candidum*).

The action of the lipase, its stability and rate of reaction are influenced by many factors, including temperature, pH, type of solvent, water activity and whether it is in an immobilized or free form (Valivety *et al.*, 1994; Soumanou *et al.*, 1999; Ma *et al.*, 2002, Rousseau and Marangoni, 2002). Liquid butteroil by itself can act as a solvent as well as a substrate and interesterification is enhanced in the presence of an organic solvent such as hexane (Lee and Swaisgood, 1997).

#### 8.4.1.2. Enzymic Interesterification of Milk Fat

Interesterification of milk fat has been carried out by various free and immobilized lipases in both solvent and solvent-free systems.

Safari *et al.* (1993) examined the interesterification of milk fat by the lipase from *Rhizomucor miehei* in various organic solvents (hexane, hexane-chloroform (70:30, v/v), and hexane-ethylacetate (70:30, v/v)). The addition of chloroform or ethyl acetate to hexane increased lipase activity. It was suggested that the polarity of the solvent influences the partitioning of water in the system with consequent effects on enzymic activity. Bornaz *et al.*

(1994) examined lipase-catalyzed interesterification of milk fat in a solvent-free system. It was found that the use of a 1,3 specific lipase from *Rhizomucor miehei* in a stirred batch reactor to effect interesterification raised the solid fat content at 20°C from ~21 to ~46% after ~48 h.

Others have used enzyme treatment of milk fat to produce modified milk fat with improved nutritional properties. *Rhizomucor javanicus* lipase immobilized onto hydrophobic hollow fibres was found to have a reduced specificity towards short chain fatty acids (Balcão and Malcata, 1998 a, b). Balcão *et al.* (1998a) examined the selective hydrolysis and interesterification of milk fat by the 1,3-specific lipase from *Rhizomucor javanicus* immobilised onto hydrophobic hollow fibres. Using a solvent-free system under controlled water activity conditions at 40°C, it was possible to produce modified milk fat with 10.9% less lauric, 10.7% less myristic and 13.6% less palmitic acid than the unmodified milk fat. This was achieved by combined hydrolysis and interesterification and recycling of the milk fat through a hollow-fiber bioreactor (Balcão *et al.*, 1998a). The modified milk fat had 2.2% less total saturated triacylglycerols, 5.4% more total monoene triacylglycerols and 2.9% less polyene triacylglycerols. As expected, the changed triacylglycerol composition of the interesterified milk fat was accompanied by a modification of the melting properties of the fat.

#### 8.4.1.3. Enzymic Interesterification of Fat Blends Containing Milk Fat

The esterification of fat blends containing milk fat provides many possibilities for widening the application range of the final product.

##### 8.4.1.3.1. Milk fat–canola oil blends

Enzymic interesterification of butterfat/canola oil blends was carried out using *Rhizopus arrhizus* lipase immobilized onto a polypropylene support in a liquid/solid two-phase system (Rousseau and Marangoni, 1998 a, b). Interesterification caused a decrease in C<sub>32–42</sub> and C<sub>54</sub> and an increase in C<sub>46–52</sub> triacylglycerols. Interesterified blends had a lower solid fat content than non-interesterified blends over the temperature range 5–40°C. Storage and loss moduli, measured by dynamic oscillation, were lower for the interesterified blends but the reverse trend was observed for the hardness index measured by cone penetrometry. Differences in trends by large (cone penetrometry) or small-scale (oscillation) deformations may be explained by the differences in the methods used for measurement. Other work has shown that fats with a similar solid fat content can also have different textural properties (Rousseau *et al.*, 1996c; Rousseau and Marangoni, 1998 a, b).

Ainsworth *et al.* (1996) showed that the interesterification of a 70% milk fat: 30% canola oil (w/w) blend using an immobilised Lipozyme IM 60

reduced the solid fat content at all temperatures between 0 and 30°C. However, the decrease in solid fat content varied with reaction time. With short reaction times, the decrease was more marked than with longer reaction times. This was correlated to initial rapid 1,3-interesterification, followed by the slower randomization of the fatty acids within the triacylglycerols.

#### 8.4.1.3.2. Milk fat–palm stearin blends

Interesterification of milk fat with palm stearin, the harder fraction obtained on fractionation of palm oil, provides a possible route for the production of pastry margarine and bakery products. Milk fat by itself, unless fractionated into a hard fraction, is not very suitable in these applications because it lacks sufficient solids at high temperatures.

Detailed studies have been carried out on the interesterification of a 60:40 blend of milk fat and palm stearin by nonspecific lipases (from *Pseudomonas* sp. and *Candida rugosa*) or 1,3-specific lipases (from *Aspergillus niger*, *Rhizomucor miehei*, *Rhizopus javanicus*, *Rhizopus niveus* and *Alcaligenes* sp.) in a solvent-free system (Lai *et al.*, 2000 a, b). Different enzymes produced interesterified products with varying degrees of interesterification and solid fat contents. The highest degree of interesterification was obtained with the non-specific lipase from *Pseudomonas* (33.9%), followed by that interesterified with the 1,3-specific lipase from *Rhizomucor miehei* (32.3%). The effect of lipase on the fat crystal polymorphs was reflected in the differences in the relative proportions of  $\beta'$  to  $\beta$  crystals in the interesterified fat blend. The *Pseudomonas* lipase-catalyzed interesterified milk fat–palm stearin blend had only the  $\beta'$  crystal form whereas the blend interesterified with *Rhizomucor miehei* lipase had a predominance of the  $\beta'$  over the  $\beta$  form. The  $\beta'$  form is desirable for applications in industrial shortening or a pastry margarine, thus making the *Pseudomonas* lipase-catalyzed interesterified milk fat–palm stearin blend suitable for these applications (Lai *et al.*, 2000 a, b).

One of the limitations encountered with the use of physical blends of different fats is incompatibility of the fats causing softening of fats due to eutectic effects. Nor Hayati *et al.* (2000) demonstrated that the eutectic effects observed in physical blends of milk fat and palm stearin was reduced on interesterification of the blend by a 1,3-specific enzyme (Lipozyme). The interesterified blend has better functionality for bakery products than milk fat.

#### 8.4.1.3.3. Milk fat–palm kernel olein blends

Milk fat may be softened by interesterification with a lauric fat (palm kernel olein, the liquid fraction from fractionated palm kernel olein).

Interesterification of blends of milk fat and palm kernel olein by a mycelium-bound lipase from *Rhizomucor miehei* or a commercially immobilized enzyme preparation resulted in a lower slip melting point and solid fat content. An interesterified product made from a 70:30 mixture of palm kernel olein and anhydrous milk fat was considered to be suitable for use in ice cream (Liew *et al.*, 2001).

#### 8.4.1.4. Enzymic Interesterification of Milk Fat with Free Fatty Acids

The interest in interesterifying milk fat with free fatty acids is due to its potential for incorporating nutritionally-desirable fatty acids into the triacylglycerol molecules. For example, to improve the fatty acid composition of milk fat for incorporation into infant formula, milk fat was esterified with eicosapentaenoic acid, dodecahexaenoic acid, oleic acid and linoleic acid using immobilised *Rhizomucor miehei* lipase (Christensen and Holmer, 1993). More targeted nutritional compositions may be obtained by controlling the pool of fatty acids added to milk fat and the conditions of interesterification. It should be noted that changes in fatty acid composition would be accompanied by an alteration of the physical properties of the fat.

##### 8.4.1.4.1. *Interesterification with oleic acid*

Various authors have examined the properties of interesterified milk fat-oleic acid. Balcão *et al.* (1998b) used a 1,3-specific lipase from *Rhizomucor javanicus* immobilised onto hydrophobic hollow fibres in a solvent-free system. The interesterification of the milk fat-oleic acid mixture (50%, v/v, oleic acid in melted milk fat) reduced the total trisaturated triacylglycerols by 27% (Balcão *et al.*, 1998b). This was accompanied by a decrease in the hypercholesterolemic fatty acids-lauric acid (8% decrease), myristic acid (6% decrease) and palmitic acid (6% decrease) and a small degree of hydrolysis. The levels of shorter chain fatty acids, which do not have a role in increasing cholesterol levels, were not affected.

Oba and Witholt (1994) interesterified milk fat with oleic acid by a commercial lipase from *Rhizopus oryzae* immobilised on glass beads. The resulting milk fat, which had been interesterified with oleic acid, had 50% more oleic acid and significantly less palmitic acid, but the level of short-chain fatty acids was not altered. As expected, the melting point of the resulting fat was lower than that of unmodified milk fat

##### 8.4.1.4.2. *Interesterification with $\omega$ -3 fatty acids*

$\omega$ -3 fatty acids are increasingly seen as components with an important role in the prevention of many diseases including coronary heart disease and

inflammatory disorders (Michelsen *et al.*, 2001). Kim *et al.* (2002) achieved 24% incorporation of  $\alpha$ -linolenic acid (from perilla oil) into butterfat in 12 h. *Rhizopus arrhizus* lipase immobilised on calcium carbonate was used to catalyze the esterification at 55°C of a reaction mixture with 1:1 molar ratio of substrate, 0.3%, w/w, water and 4%, w/w, of enzyme. Incorporation of  $\alpha$ -linolenic acid into butterfat was accompanied by improved spreadability of the fat compared to butterfat.

#### 8.4.1.4.3. *Interesterification with conjugated linoleic acid*

Conjugated linoleic acid (CLA) may have a role in improving the nutritional and health properties of milk fat (Wahle *et al.*, 2004; see Chapter 3). A range of lipases was compared for their efficacy of catalysing the incorporation of CLA into milk fat in solvent-free systems (Garcia *et al.*, 2000). It was concluded that it was technically feasible to incorporate CLA into milk fat with the use of immobilised *Candida antarctica* lipase. However, the nutritional benefits and physical properties of the CLA-enriched milk fat need to be investigated prior to consideration of this technology for industrial application.

#### 8.4.1.5. Comparison Between Chemical and Enzymic Interesterification

Similar changes in chemical composition and melting properties were reported for chemical and enzymatic interesterification of milk fat with a non-specific lipase (Kalo *et al.*, 1986 a, b). Both processes result in randomisation of the fatty acids.

When a 1,3-specific lipase is used for interesterification, the enzymatically-modified product has some different properties compared to those of a chemically-interesterified product. For example, the dropping point of butterfat was increased slightly by chemical interesterification whereas interesterification by a 1,3-lipase from *Rhizopus arrhizus* led to a 2–4°C decrease in dropping point. Although both methods of interesterification reduced hardness, the magnitude of the effect was greater for the enzymatically-interesterified fat (Marangoni and Rousseau, 1998).

When 80:20 blends of butter fat and canola oil were used, chemical interesterification increased the solid fat content above 10°C while enzymatic interesterification by *Rhizopus arrhizus* lipase reduced solid fat content over the range 5–40°C (Rousseau and Marangoni, 1999).

### 8.4.2. Enzymic Hydrolysis

A number of studies have examined the use of lipases from a variety of sources for hydrolysis of milk fat. Lipase from *Aspergillus niger* could be

directed to hydrolyze butyric acid preferentially in butter-in-oil emulsions, thus enhancing the production of free fatty acids responsible for dairy flavor (Garcia *et al.*, 1991). In organic solvent-free systems, lipase from *Candida rugosa* had an increased activity toward butyric acid compared to lipase from *Pseudomonas fluorescens* (Marangoni, 1994), indicating an opportunity to effect selective changes in milk fat hydrolysis by an appropriate choice of enzyme.

Alternatively, the enzyme may be used in an encapsulated form. Chen and Chang (1993) showed that hydrolysis of milk fat by lipase from *Candida cylindracea* encapsulated in reverse micelles formed by soybean lecithin in isoctane, could be manipulated to favor the release of short-chain fatty acids by using a higher concentration of enzyme and a higher ratio of water to surfactant concentration at 45°C.

## 8.5. Cholesterol Reduction

Cholesterol is present in milk at a level of 0.25–0.46%. The interest in removing cholesterol from milk fat has been driven primarily by consumer concern about the possible link between cholesterol and heart disease. Although there is still some debate about the causal relationship between dietary cholesterol and heart disease, a marketing position has been created for low-cholesterol products and this has spurred interest in examining alternative ways of cholesterol removal in the 1980s and 1990s (Schlimme, 1990). A number of physical, chemical and biological processes have been used to reduce the level of cholesterol in milk fat (Boudreau and Arul, 1993). Cholesterol-reduced butter has been introduced on the market in Europe (Anon, 1992).

### 8.5.1. Distillation Processes

Distillation processes exploit the low volatility of cholesterol compared to the major triacylglycerols of milk fat for removal of cholesterol. Vacuum and short-path molecular distillation processes can efficiently remove cholesterol but it may be achieved at the expense of removing some low-molecular weight triacylglycerols and flavor components of the milk fat. Vacuum steam distillation is commonly used for refining fats and can also be used to refine milk fat. Cholesterol-reduced milk fat, which was produced by steam distillation, has been used successfully to formulate butter, cream and ice cream (Schroder and Baer, 1991, Elling *et al.*, 1995, 1996). If the flavor of milk fat is to be preserved, the flavors can be trapped and re-incorporated into the milk fat that has been stripped of cholesterol (Boudreau and Arul, 1993).

### 8.5.2. Supercritical CO<sub>2</sub> Extraction

Supercritical CO<sub>2</sub> extraction has potential for the removal of cholesterol. Careful manipulation of process conditions is necessary to obtain efficient removal of cholesterol. When supercritical CO<sub>2</sub> extraction is used to fractionate milk fat, the liquid fraction is enriched in cholesterol while that of the solid fraction is reduced (Arul *et al.*, 1988b).

#### 8.5.2.1. Process Conditions

The efficiency of supercritical CO<sub>2</sub> for removing cholesterol is temperature- and pressure-dependent. Removal of about 90% cholesterol from milk fat was achieved using bench-scale supercritical CO<sub>2</sub> extraction using an ascending pressure profile (Bradley, 1989). With multistage supercritical CO<sub>2</sub> extraction, more than 90% cholesterol can be removed from milk fat (Anon, 1989).

#### 8.5.2.2. Use of Adsorbents During Supercritical CO<sub>2</sub> Extraction

The use of adsorbents in conjunction with appropriate conditions for supercritical CO<sub>2</sub> extraction enhances the efficiency of cholesterol extraction. Selective removal of 97% of cholesterol has been achieved with the use of silica gel as an in-line adsorbent (Huber *et al.*, 1996). Removal of 96% of cholesterol in milk fat fractions can be achieved by a combined supercritical CO<sub>2</sub> extraction and an alumina adsorption process (Mohamed *et al.*, 1998).

### 8.5.3. Treatment with Adsorbents

Treating milk fat with adsorbents (e.g., carbon, activated carbon, carbon impregnated with metal salts, porous glass, impregnated or chemically-bonded porous glass) can reduce the cholesterol content of milk fat (Keen, 1991). These adsorbents also remove colours and flavors, but these may be added back into the butterfat.

Cholesterol may be removed selectively by cyclodextrins. Melted milk fat is mixed with a cyclodextrin solutions and the complex is removed by washing. The ability of cyclodextrin to form an inclusion complex with cholesterol has been applied to reduce the cholesterol content of milk fat, cream or milk (Courregelongue and Maffrand, 1989; Oakenfull *et al.*, 1991). However, the high cost of cyclodextrin prevents its use on an industrial scale.

Alternative adsorbents for cholesterol are food-grade saponins. Complexation of cholesterol in milk fat with saponins in aqueous solutions, followed by separation of the cholesterol-saponin complex has been shown to be technically feasible for cholesterol removal (Sundfeld *et al.*, 1993).

Micich *et al.* (1992) demonstrated that polymer-supported saponins could be used to remove cholesterol from milk fat and that the polymers could be regenerated by solvent extraction without loss of cholesterol-binding capacity.

#### 8.5.4. Treatment with Enzymes

Alternatively, enzymes may be used to convert cholesterol into other products (Boudreau and Arul, 1993). Enzyme systems that have been examined include (1) cholesterol reductase, which converts cholesterol to coprostanol, a product that is poorly adsorbed by the body, and (2) cholesterol oxidase, which oxidises cholesterol to non-steroid compounds. In the latter case, the oxidised products are toxic (see Chapter 18). When enzymes are used, it is necessary to demonstrate both the efficient conversion of the cholesterol as well as the safety of the reaction products.

### 8.6. Future Trends

The food industry is moving away from the use of chemical processes towards physical processes because of consumer perceptions, safety and environmental concerns. Of the physical processes used in the milk fat industry, dry fractionation has emerged as the preferred method for modifying the properties of the fat as it has the advantage of preserving its delicate flavor. Dry fractionation is likely to remain the dominant process for modification of milk fat in the near future (Timms, 2005). Optimising crystallization for maximum functionality and blending with other oils and fats will also remain important areas of focus. Noshu *et al.* (2004) could produce finer, more stable fat crystals for pastry margarines, giving better quality pastry by using high-pressure surface heat exchangers up to 45 MPa.

Much higher pressures are used in high-pressure processing, which is now a commercial reality in the food industry, with more than 65 commercial installations in 2005. With more research, the potential for this technology for milk fat differentiation may be realised. Buchheim and El-Nour (1992) showed that exposure to pressures of 100–400 MPa for 15 min induces milk fat crystallization. Recent work has demonstrated that high-pressure also induces shifts in the melting and crystallization of bulk anhydrous milk fat and milk fat fractions. A shift of phase transition by  $\sim 16^{\circ}\text{C}$  per 100 MPa for milk fat was obtained from isobaric heating and cooling (Frede and Buchheim, 2000). The use of high-pressure processing and other emerging food processing technologies (e.g., ultrasonication) for modifying the milk fat component in isolation or when it is part of a food product has not yet been fully explored.

Solvent-free enzymatic interesterification of milk fat alone or with other fats or fatty acids provides the most acceptable route for modification of the triacylglycerol structures in milk fat and further research and development in this field is expected to provide physical and physiological benefits. From a nutritional perspective, it is of interest to examine the effects of randomized milk fat on serum cholesterol. Christophe *et al.* (1978) reported that substitution of native milk fat with chemically-randomized interesterified milk fat reduced cholesterol levels in man. However, others found that there was no effect on serum cholesterol levels in man as a result of substitution of enzymatically randomized milk fat (De Greyt and Huyghebaert, 1995). Further studies are required to determine if interesterified milk fat provides a nutritional benefit.

There are opportunities for using on-farm and post-farm approaches for manipulating milk fat to improve its attractiveness as an ingredient in functional foods (Baer *et al.*, 2001; Hillbrick and Augustin, 2002). The emergence of the functional foods (i.e., foods that provide benefits beyond normal nutrition) and the accumulating evidence that some fats, minor lipids and fat-soluble products can have important roles in nutrition and the prevention of disease (Parodi, 2003, 2004; see Chapter 17) has opened opportunities for milk fat and its components in the growing market for functional foods.

## Acknowledgements

The authors would like to thank Christine Margetts for her contributions and helpful comments on the manuscript.

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# Chemistry and Technology of Butter and Milk Fat Spreads

M.K. Keogh

## 9.1. Introduction

### 9.1.1. Legislation

Butter, margarine and spreads are viscoelastic solids (i.e., plastic emulsions that remain solid at room temperature and are mainly of the water-in-oil type). EU regulation (EC) No. 2991/94, in force since January 1996, provides definitions of spreadable fat products in the European Union. Butter, margarine and (full-fat) blends must contain not less than 80% fat but less than 90% fat, a maximum of 16% water and 2% non-fat milk solids. The salt content must be indicated on the label. Reduced-fat or three-quarter fat products should contain more than 41% but not more than 62% fat. Low-fat, light or half-fat products should contain 10–41% fat. Margarine should not contain more than 3% milk fat. Blended milk fat-vegetable fat products should have a milk fat content of 10–80% of the total fat. The milk fat may be modified only by physical processes, which in effect permits the use of thermally fractionated milk fat. It is also possible to produce zero-calorie fat spreads in which either fat substitutes or enzyme-resistant zero-calorie lipid-like materials, such as sucrose polyesters, are used (Mattson *et al.*, 1971). The development of low-fat spreads was first stimulated in the US by fat shortages during World War II. Oil-in-water spreads were the first to appear, in the 1950s and 1960s, but they had a number of technical problems. Water-in-oil spreads were first reported by Bullock (1966) but they did not gain a significant share of the edible fats market in the US (Behrens, 1988).

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

### 9.1.2. Emulsion Stability

Oil-in-water emulsion products, such as milk, cream and ice cream, are stabilized by homogenization to reduce the mean fat globule size of the dispersed phase. Water-in-oil emulsion spreads are stabilized by shearing to reduce the water droplet size and by simultaneous cooling to entrap the dispersed water droplets in the continuous phase of plastic fat, mainly in the form of small  $\beta'$  crystals. This technology, called scraped-surface cooling, is used to produce margarines, reduced-fat and low-fat spreads. Butter-making involves churning (i.e., shearing to break partially the oil-in-water emulsion of cream to concentrate the fat to 80–82% by removing the aqueous phase as buttermilk). Thus, buttermilk is essentially skim milk enriched in fat globule membrane material (McCarthy and Headon, 1979). Further churning reduces the level of the aqueous phase to 16% water, containing 2% solids non-fat and up to 2% added salt. The dispersed water droplets are further sheared in the so-called working stage to reduce their diameter to less than 20  $\mu\text{m}$  (Muller, 1952), to prevent microbial growth and the appearance of visible moisture. Dairy and blended spreads containing more than 72% fat can be manufactured using either scraped-surface cooling or churning equipment. Efforts by equipment manufacturers to produce low-fat butter using continuous churning equipment by incorporating the extra aqueous phase using an additional shearing unit have been commercially successful (Norgaard *et al.*, 1990). High-fat products (>95% fat), such as baking fats, are also processed using scraped-surface or drum cooling but the primary aim in their manufacture is the production of small  $\beta'$  fat crystals for optimum functionality of these fats in baked goods (Joyner, 1953; Hoerr, 1960; Brooker, 1993). The principal ingredients of fat spreads are fat, emulsifier, milk protein, stabilizer, sodium chloride and water. Each of these affects the emulsion and processing characteristics of the final product. Other ingredients, such as preservatives, colors, and flavors, do not affect product processability. In dairy spreads, the fat source is milk fat to which can be added hard (Keogh *et al.*, 1988) or soft milk fat fractions (Verhagen and Warnaar, 1984). In margarine spreads, the fat source is usually either soybean oil or sunflower oil blended with a hydrogenated vegetable oil, typically in the ratio 3:1. Blended spreads contain a mixture of milk, vegetable, and other animal or marine fats.

### 9.1.3. Consumer Pressures for Change

A number of economic (price, inadequate promotional expenditure, low retail margins, lack of both brand advertising and innovations in packaging), medical (COMA Report, 1984; Surgeon General's Report,

1988; Department of Health (Ireland) Report, 1991) and social (more sedentary lifestyle, breakdown in traditional meal patterns, decline in the level of home cooking, wider choice of foods, decrease in bread consumption, and increased use of refrigerators, which highlighted the poor spreadability of butter) factors, have contributed to the decline in butter consumption. The development of spreads has been the response by food manufacturers to these market forces.

Consumer pressures for a cold-spreadable and more nutritionally acceptable spread were met in Sweden in 1970 by the introduction of Bregott<sup>™</sup>, an 80:20 butterfat/soya oil blend, and the low-fat spread, Latt and Lagom<sup>™</sup> (60:40 butterfat/soya oil), in 1974. It is possible to produce a product that is not subject to excessive softening or oiling-off at high ambient temperatures by using approximately 50% milk fat, 25% vegetable oil and 25% hydrogenated vegetable oil. Such a blended product, called Clover<sup>™</sup>, was first marketed in the UK in 1983. This product had a monopoly in the UK until 1985 when Meadowcup<sup>™</sup> and Golden Churn<sup>™</sup> were launched by Kerrygold and Kraft, respectively. The Dairygold<sup>™</sup> blended spread was launched on the Irish market in February 1985. The first low-fat margarine in the UK was Outline<sup>™</sup>, which was marketed by van den Berghs in the early 1970s. The first low-fat blend was Gold<sup>™</sup>, which was launched in 1979 by St. Ivel (ERC Statistics International Ltd., 1987). Initially, this product was a blend of milk fat and soya oil but soon after its introduction was changed to a full vegetable oil-based product. The first low-fat product in Ireland was Dawn Light<sup>™</sup> butter produced by Kerry Group plc. Reduced-fat butter (Kerry Group plc), reduced-fat blend (Lifestyle, by Glanbia plc), low-fat margarine (Low, by Kerry Group plc) and low-fat butter from cream (Connaught Gold<sup>™</sup>, by North Connaught Farmers Co-op) are also manufactured in Ireland.

The most important developments of recent years have been in nutritional spread products. An olive oil-based product (Golden Olive, by Kerry Group plc), a vegetable oil product containing 8–13.5% plant stanol esters (Benecol, by Raisio Benecol Ltd, Raisio; Duchateau *et al.*, 2002; Louter *et al.*, 2002) or 14% sterol esters (Flora Pro-Active, by Unilever plc, London) and a spread containing probiotic bacteria (Tesco own-brand by Kerry Group plc; Stanton, 2003) are being marketed. An experimental 20% fat spread containing 7.5% inulin as prebiotic has also been suggested (Rooyackers *et al.*, 1994), but, to date, claims of prebiotics in any commercial product have not been made.

## 9.2. Technical Aspects of Butter Manufacture

### 9.2.1. Chemical and Physical Principles

The manufacture of butter can be divided into five steps:

1. Concentration of the fat phase of milk by mechanical separation.
2. Crystallization of the fat phase of cream by cooling.
3. Phase inversion of the oil-in-water emulsion of cream by shearing.
4. Removal of buttermilk by drainage.
5. Formation of a viscoelastic water-in-fat emulsion by working.

Each of these steps is partial and some of the steps may be combined in some processes. Only the basic principles are outlined here; for further details, the reader is referred to manuals such as Anderson (1986) and Bylund (1995).

The fat content of cream is typically in the range 30–55% for batch churning systems but in a narrower range of 38–42% for continuous systems. The cream is pasteurized at 85–110°C for 10–30 s, which results in the survival of few microorganisms. Because of the high temperature treatment, the cream acquires a beneficial cooked flavor and some antioxidant properties due to the activation of free sulphhydryl groups in the whey proteins. Some gas bubbles and any volatile odors present are also eliminated. Alternatively, the cream may be fermented (ripened or soured) after pasteurization using a culture of *Lactococcus lactis* subsp. *lactis*, *Ln. lactis* subsp. *cremoris* or a citrate-positive strain of *Lactococcus lactis* to reduce the pH to 4.5–5.0 by generating lactic acid. *Leuconostoc mesenteroides* subsp. *cremoris* or *Ln. mesenteroides* subsp. *citrovorum* simultaneously produce other desirable flavor compounds, principally diacetyl (1–3 mg/kg). In the process developed at the Netherlands Institute for Dairy Research (NIZO) (van den Berg, 1982), instead of fermenting the cream, flavor compounds and lactic acid from selected starters and starter permeate, respectively, are injected into the butter at the working stage.

The cream is cooled to 2–5°C for at least 4 h (ideally 24 h) to allow crystallization of the fat. At 5°C, the solid fat content of milk fat, determined by NMR, is in the range 50–60%.

Butter-making by churning (steps 3–5 above) involves shearing at a low temperature, which partially breaks the oil-in-water emulsion of the cream and after drainage of the buttermilk, concentrates the fat to 80–82%. After drainage of most of the buttermilk, the butter is sheared further (worked) to produce a viscoelastic water-in-fat emulsion. The objective is to reduce the aqueous phase to a maximum of 16% water, containing 2% solids non-fat with the optional addition of up to 2% salt and/or acid/flavor concentrates.

The controlling variables of the churning process are:

1. Fat content, globule size and pH of the cream
2. Cream temperature, time for crystallization and wash water temperature
3. Shear rate (agitation speed, degree of churn fill, dimensions and shape of churn)

The aim should be to control all the process variables within narrow ranges, so that the butter-making process is consistent.

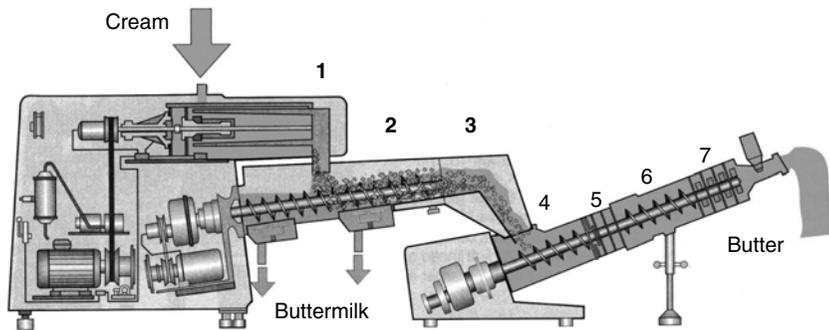
A high fat content in the cream aids inversion of the emulsion, facilitating churning. The breed of cow and the stage of lactation are the main factors that affect fat globule size. Fat globules are larger and churning is easier in cream from Jersey/Guernsey milk and from milk from herds in early to mid-lactation than in late lactation. As the pH decreases, the fat globules membranes become weaker, so that fermented cream is more easily churned than sweet cream.

The churning temperature (typically 4–5°C for batch or 8–9°C for continuous churning) and the degree of fat crystallization affect most aspects of the process. A high churning temperature reduces churning time, increases fat coalescence and the ratio of free to globular fat, which in turn affects the visible moisture, texture, firmness and color of the final product. The churning time and the degree of fat coalescence affect the size of the butter grains, which should be in a certain range for optimal results. Small grains can be lost in the buttermilk, which typically contains 0.45–0.65% fat. Smaller grains also have a larger surface area, which adsorbs large amounts of aqueous phase on the surface. Conversely, large grains hold too much aqueous phase within the grains. Larger grains have a smaller surface area, making it difficult to incorporate added salt. The texture of the butter can vary from soft, crumbly or brittle to sticky or greasy due to the ratio of free to globular fat. The degree of working affects visible moisture, surface moisture, color and texture.

The shear rate during churning is governed by the speed of agitation, degree of churn fill, churn dimensions and shape. When cream containing crystallized fat is agitated during whipping or churning, fat globules form clusters, which subsequently surround the incorporated air bubbles. Some of the fat globules break due to friction and liberate their fat as free fat, which in turn destabilizes the foam, leaving buttermilk surrounding, or incorporated into, the butter grains. Higher temperatures result in more free fat, which causes the foam to break more easily. The higher the proportion of free fat, the more greasy the butter at high temperature and harder at low temperature. As churning progresses, more buttermilk is incorporated into the butter grains, thus reducing the fat lost in the buttermilk. These dispersed

water droplets are sheared further during working to reduce their maximum diameter to less than  $20\ \mu\text{m}$  (Muller, 1952), to prevent microbial growth and the appearance of visible moisture.

In continuous butter-making machines (Figure 9.1) that are enclosed, altering the flow rate of the cream pump and the speed of the agitation shaft (1000–2500 rpm) affects the churning time. The moisture content of butter reaches a minimum at a certain intermediate speed. At the end of churning cylinder 1, butter grains begin to appear. Optimum grain size is obtained in cylinder 2 by altering the speed of the paddles or augers from 20 to 90 rpm. After removing part of the buttermilk through outlets in cylinder 2 (or at the front of the inclined cylinder 4 in some machines), the butter grains and remaining buttermilk are sheared in the working sections by variable speed augers 4 and 6. The moisture content of the butter increases with auger speed. Secondary drainage of buttermilk can be facilitated through small perforations in the base of the worker cylinders under the augers. The butter is finally forced through a series of perforated plates in cylinder 7, when salt slurry/acid/flavor compounds may be injected after the first plate, followed by final working through the remaining plates. Typically, the moisture content after the first perforated plate is 14%, so that 2% water and 2% salt can be added as a 50% salt slurry to give butter of the desired final composition. In the NIZO process, in which acid and flavor compounds are added, the maximum moisture content at this stage is 13.5%.



**Figure 9.1.** Diagram of a continuous butter-making machine. Churning cylinder 1; separating section 2; squeeze-drying section 3; second working section 4; injection section 5; vacuum working section 6; final working stage, 7. [From Bylund, G. (1995). Butter and dairy spreads. In: *Dairy Processing Handbook*, 2nd edn, p. 274, Tetra Pak Processing Systems AB, Lund, Sweden.]

In continuous churns, the working force,  $F$ , used to shear the butter can be expressed as  $F \propto \eta \cdot dv/dx$ , where  $\eta$  = viscosity of the butter and  $dv/dx$  is the velocity gradient. At constant viscosity,  $F \propto dv/dx$  (i.e., proportional to the throughput rate and inversely to the size of holes in the perforated plates), since viscosity and the velocity gradient are inversely related. In countries with marked seasonality of milk supply (e.g., Ireland and New Zealand) the viscosity of cream is higher in winter than in summer since the fat globules are smaller and more numerous and the solid fat content is higher. In winter, cream temperature can be increased by 1°C to reduce cream viscosity, thereby increasing the velocity gradient and the degree of working of the butter.

After churning and during packing, the temperature of the butter should not exceed 14°C (Anon, 1978), because slow crystallization and further structure development during cold storage will lead to a firmer product. Aluminum foil-laminated materials are used to wrap butter, as they fully protect the fat from light-induced oxidation and associated off-flavor development.

### 9.3. Technical Challenges in the Processing of Fat Spreads

#### 9.3.1. Rates of Microbial Growth

Fat spreads, whether of the high-fat or low-fat type, are usually water-in-oil plastic solids (Bullock, 1966). Microorganisms that survive the heat treatment applied prior to scraped-surface cooling are confined by the small size of the water droplets. In butter, living bacteria were not observed in droplets less than 20  $\mu\text{m}$  in diameter (Muller, 1952). Poorly-worked spreads that supported bacterial growth, had moisture droplets ranging from 50 to 100  $\mu\text{m}$  in diameter. Some growth of microorganisms is expected to occur in droplets between 20 and 30  $\mu\text{m}$  diameter, depending on the size and oxygen requirements of the organism but droplets should, in general, not exceed 30  $\mu\text{m}$  and ideally be less than 20  $\mu\text{m}$  in diameter (Bullock and Kenney, 1969). The bacteriostatic effect of 1% NaCl in a 40% fat spread containing about 53% water is much less than 2% NaCl in an 80% fat butter containing 16% water. In contrast, microorganisms are relatively free to grow and multiply in oil-in-water spreads, which have a different texture and melt-down and lack freeze-thaw stability (Tobias and Tracy, 1958; Weckel, 1965; Seas and Spurgeon, 1968).

The poor correlation between bacterial growth rate and droplet size is due partly to the limitations of the light microscope method used (Dolby, 1965). Other influencing factors are nutrient level, salt and pH of the droplet

contents. The light microscopy method involves viewing a sample of the spread under a cover glass, which distorts to some extent the droplet appearance at the spread surface. More recent methods (Juriaanse and Heertje, 1988), using electron microscopy after exposure of a fresh internal surface of the spread by freeze-fracturing, are more accurate. These latter methods have been coupled with image analysis and counting techniques, which give a true distribution of droplet size. However, because of the commercial value of these results, nothing has been published on the application of the combined techniques to fat products (Brooker, 1992 personal communication). Electron microscopy has been used successfully to study the fat crystal shell surrounding the droplets (Heertje *et al.*, 1987; Chawla *et al.*, 1990) and to determine the ion content of the droplets using X-ray spectroscopy after electron bombardment of the spread surface (Brooker, 1990).

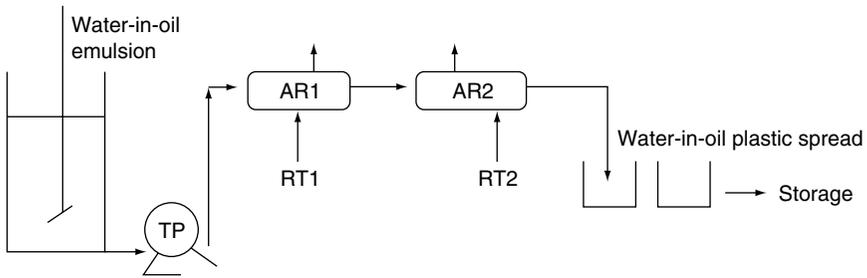
### 9.3.2. Phase Inversion

It has been shown by light microscopy (Keogh *et al.*, 1988) and by scanning electron microscopy with freeze etching (Brooker, 1990) that if droplets exceed 30  $\mu\text{m}$  in diameter, some will join together to form channels or 'lakes'. Eventually, the water phase may become continuous during processing (i.e., inversion of the emulsion may occur). At the other extreme, if the water droplets are too small or are over-stabilized, the phase inversion required in the mouth under the influence of shear and added aqueous phase (saliva) will not take place, or will occur too slowly. Such spreads will have an unpleasant gummy mouth-feel. The cooling sensation due to the effect of melting fat will also be absent.

## 9.4. Technology of Spread Manufacture

### 9.4.1. Processing

A scheme for the continuous processing of fat spreads is outlined in Figure 9.2. This comprises two steps, namely preparation by stirring of an aqueous phase-in-oil emulsion, followed by pumping the emulsion at a certain throughput (TP) using one or two scraped-surface coolers in series at a defined agitation rate (AR) and at a defined refrigerant temperature (RT) to form a plastic fat product with dispersed water droplets. Pin-working or crystallizing units are usually sited after each scraped-surface unit. In these units, the product is sheared, without cooling, by pins on the central rotating shaft and by static pins on the inner wall of the cylinder. The process variables, TP, AR and RT, have been studied on a pilot-scale plant (Keogh *et al.*, 1988). To produce a high-moisture spread of the water-in-oil type, the emulsion must



**Figure 9.2.** Line diagram showing variables for the production of fat spreads

- TP: Pump throughput
- AR1: Agitation rate, scraped surface cooler no.1
- AR2: Agitation rate, scraped surface cooler no.2
- RT1: Refrigerant temperature, scraped surface cooler no.1
- RT2: Refrigerant temperature, scraped surface cooler no.2

be worked more intensively (850–1050 rpm) at a higher refrigerant temperature (−2°C to +4°C) and at a lower throughput (maximum 75 kg/h) than margarine. Under-working or over-working causes inversion of the emulsion. When softer fats than milk fat are used for processing, optimum results are obtained at a lower throughput. Buchheim and Frede (1994) reviewed other variations of the butter churning and margarine scraped-surface cooling technologies. Frede and Buchheim (1994) reported valuable observations, based mainly on microscopy, on cultured butter, cream tempering (physical ripening) and cream destabilization (phase inversion). It was demonstrated that butter made by the churning process could be produced with a spreadability close to that of a soft margarine, using a cream prepared by blending low-melting and high-melting point milk fat fractions in the ratio 60:40. Hoffmann (1989) has reviewed the role of the scraped-surface crystallizing units and resting tubes used for margarines and shortenings. A chapter on margarine and shortening technology by Bell (1991) concentrated on historical developments while those by Munro *et al.* (1992) and Lane (1992) were devoted to milk fat products.

Technical and product information is also available from the three main suppliers of scraped-surface equipment in Europe, namely Gerstenberg and Agger (Denmark), Schröder (Germany) and Johnson (UK) who fabricate Perfector, Kombinator and Votator equipment, respectively. Numerous patents on fat spreads have also been published, but their review is outside the scope of this chapter.

## 9.5. Fundamental Aspects of Emulsions

### 9.5.1. Emulsions: Theory, Rheology and Stability to Inversion

An understanding of the mode of action of the component ingredients of fat spreads during processing requires a fundamental knowledge of the theory, rheology and stability—especially to inversion—of emulsions.

#### 9.5.1.1. Emulsion Theory

Liquid emulsions are inherently unstable to a varying degree. It is important to understand, therefore, the mechanisms that contribute to emulsion stability. Before the solidification step, instability of an emulsion can arise due to either phase separation or phase inversion (Mulder and Walstra, 1974). It is evident that the likelihood of phase inversion will increase as the fraction of dispersed phase increases. The vast majority of literature references are concerned with the stability to phase separation as coalescence or creaming in oil-in-water emulsions (Halling, 1981; Jaynes, 1983). In addition, a method for determining the stability of water-in-oil emulsions to inversion has not been reported. It is usually assumed that certain aspects of oil-in-water emulsion theory apply in reverse to water-in-oil emulsions.

#### 9.5.1.2. Electrostatic or Charge Stabilization

This theory is based on the interactions between charged solid colloidal particles in an aqueous phase. The mechanism was first described in 1941 by B.V. Derjaguin and L. Landau and in 1948 by E.J.W. Verwey and J.Th.G. Overbeek and is referred to as the DLVO mechanism after them (see Bergenståhl and Claesson, 1990). Charged particles in suspension attract oppositely charged ions from the immediate environment to their charged surfaces to form a layer of counter-ions. This, in turn, leads to the formation of a further secondary layer of opposite charge to the first layer—the so-called electrical double layer. Thus, two charged particles give rise to a repulsion potential, which depends on the distance between them and the height of the potential. If the repulsion potential is greater than the attraction potential, the particles will be stable. The attraction potential the van der Waals' interaction, has as its source, the rapidly fluctuating dipole moment ( $\sim 10^{-30}$  C·m) of a neutral atom, which induces a polarized charge in neighbouring atoms, resulting in an attraction. The extent of the attraction is proportional to the polarizability of the atoms, divided by the distance between them. A feature of the theory is the rapid increase in attraction potential at small distances. The sum of the repulsive and attractive potentials

gives the total potential, which determines the stability of the system. For very short distances, the attraction potential is always the greater and aggregation results. For intermediate distances, the repulsive potential, if large, results in a stable system. The most useful aspect of the DLVO theory is its ability to explain the destabilizing effect of added neutral salts, which compress the double layer and reduce the repulsive potential, resulting in flocculation. According to the Schulze-Hardy rule, the ratio of critical flocculation concentration for monovalent, divalent and trivalent ions is 100:1.6:0.13. Thus, divalent ions are over 50 times more effective than monovalent ions in destabilizing colloids (Friberg *et al.*, 1990).

It is important to state that while the DLVO theory is a useful starting point, it applies to solid, charged particles in an aqueous suspension but does not apply accurately to emulsions, which are liquid systems. Also, the mechanism does not apply to water-in-oil emulsions, in which the repulsive layer is located on the concave side of the interface and, therefore, is too distant from a neighboring droplet to exert a repulsive force. Moreover, since the continuous phase of a water-in-oil emulsion is non-ionizing, the development of an electrical double layer is not to be expected (Sherman, 1955b). In addition, the monoglyceride emulsifiers normally used in the manufacture of spreads are non-ionic. Conversely, the charge on protein molecules is quantifiable by the zeta potential and depends on the pH of the suspending medium, as well as on other ions. However, proteins such as sodium caseinate bind cations, making the prediction of the emulsion behavior very difficult in anything more than a qualitative way. Thus, oil-in-water emulsions stabilized by  $\alpha_s(\alpha_{s1} + \alpha_{s2})$ -casein follow the DLVO theory and are reversibly flocculated by increasing ionic strength but similar emulsions stabilized by  $\beta$ -casein behave differently (Dickinson *et al.*, 1987).

### 9.5.1.3. Steric Stabilization

Flexible macromolecules, such as proteins, and small molecules, such as surfactants, are amphipathic and may form a layer at the oil-water interface. These molecules may also partly stabilize emulsions by forming a physical barrier to close contact, thereby reducing the attractive van der Waals' forces to ineffective levels (Dalgleish, 1989). Repulsion can arise in either of two ways and physico-chemical calculations are available for both mechanisms in oil-in-water systems. Either the approaching protein-coated particles will tend to compress or alternatively interpenetrate the adsorbed protein layer on adjacent particles. The optimum structure of the stabilizing protein will be dealt with in the section on protein as an ingredient.

#### 9.5.1.4. Solid Particle Stabilization

In a few instances, emulsions can be stabilized by solid particles as shown by S. U. Pickering in 1907 (see Petrowski, 1976). Examples are mustard seeds in traditional mayonnaise (Anon., 1968) or crystals of high melting-point triglycerides in margarine or butter (Precht and Buchheim, 1980; Heertje *et al.*, 1987). The solid particles are considered to act as a barrier, which prevents the coalescence of droplets. Bancroft (1913) observed that the phase that wets the solid more easily will become the continuous phase.

Traditional mayonnaise is an 80% oil-in-water emulsion, which may rely on mustard seeds for solid particle stabilization. Two constituents of egg yolk, lecithin and cholesterol, are surfactants, which promote the formation of oil-in-water and water-in-oil emulsions, respectively. The ratio of lecithin to cholesterol in egg yolk favors the water-in-oil type but the final emulsion type formed is due to the action of mustard seed, which favors an oil-in-water emulsion (Petrowski, 1976).

#### 9.5.1.5. Stabilization by Rheological Methods

Increasing the viscosity of the continuous phase in an oil-in-water emulsion increases stability to flocculation, coalescence and oiling-off by reducing the frequency of collision between droplets. However, the kinetic theory of flocculation suggests (Friberg *et al.*, 1990) that the effect of the viscosity of the continuous phase is less than expected without the concurrent energy barrier provided by electrostatic and/or steric effects. In non-food water-in-oil systems, the viscosity of the continuous phase can be adjusted by choice of hydrocarbon oil of varying molecular weight or by addition of oil-soluble compounds of high viscosity. However, in water-in-oil emulsions, there are no indications of the effects of the viscosity of the internal aqueous phase on the stability of emulsions to oiling-off or inversion (Sherman, 1950). The higher the viscosity of the aqueous phase in multi-ingredient food systems containing protein and starch, the higher the stability (Platt, 1988). In this case, stability may be due to effects associated with the viscosity generated by the ingredients (which will be referred to again below) rather than to the effect of viscosity *per se*. On the contrary, Sherman (1955a) showed that the viscosity of the aqueous phase had no effect on the viscosity of water-in-oil emulsions and, therefore, should not affect emulsion stability.

In earlier work (Sherman, 1950), inversion of water-in-oil emulsions was achieved by increasing the proportion of the dispersed aqueous phase. Emulsions containing 2–5% non-ionic emulsifier and up to 50% water are Newtonian. Above this level of water, the rheological parameters increased significantly and inversion occurred at a water concentration of 75–80%. The

higher the concentration of emulsifier, the higher the viscosity before inversion. A large decrease in viscosity accompanied inversion to an oil-in-water emulsion. The viscosity of the inverted oil-in-water emulsion should be of the same order but slightly less than that of a water-in-oil emulsion because the continuous phase dominates the viscosity at identical ratios of dispersed phase. Thus, since water has a viscosity of 1 mPa s, while that of hydrocarbon oils is of the order of 25 mPa s, the oil-in-water emulsion at the same level of dispersed phase will have a slightly lower viscosity. Since there are few other relevant references devoted to water-in-oil emulsion theory (Sherman, 1955c; 1967a; 1967b), one must return to the view (quoted in many reviews on emulsions) that oil-in-water emulsion theory applies in reverse to water-in-oil emulsions. References outside the patent literature on fat spreads are not available on multicomponent water-in-oil emulsions using food ingredients.

#### 9.5.1.6. Rheology of Water-in-oil Emulsions

According to Sherman (1955a,b), the factors that affect the rheological characteristics of a water-in-mineral oil emulsion are:

- *Volume of the dispersed phase.* Below a concentration of 50% aqueous phase and with 2–5% non-ionic emulsifier, emulsions behave as Newtonian fluids; above 50% aqueous phase, emulsions become increasingly non-Newtonian (i.e., become shear rate-dependent and develop a yield value).
- *Viscosity of the dispersed phase.* The viscosity of the dispersed phase that ranged from 11 to 560 mPa s, did not affect the viscosity or yield value of the emulsion. The dispersed phase fraction,  $\varphi$ , was 0.717.
- *Viscosity of the continuous phase.* The viscosity of the continuous or oil phase is related directly to emulsion viscosity and yield value. In non-food systems, oil viscosity can be varied by choosing hydrocarbon oils of varying molecular weight or by adding of viscous, oil-soluble compounds. In addition, as already mentioned, emulsion stability is increased by reducing the rate of globule flocculation in a continuous phase of higher viscosity.
- *Type and concentration of emulsifier.* The viscosity and yield value of emulsions ( $\varphi_{\text{vol}} = 0.66$ ) depend on the chemical nature of the emulsifier. Sherman (1955c) proposed two possible reasons for this, namely interfacial viscosity and interfacial adsorption. Interfacial viscosity affects the resistance of droplets to deformation, which is reflected in the resulting emulsion viscosity. A high level of interfacial adsorption enlarges the size of the interfacial layer significantly and increases emulsion viscosity. Adsorption of emulsifier at the interface should also increase with the concentration of emulsifier. The

increase in the overall dimensions of the resulting droplets increases the viscosity and yield value.

- *pH*. In a study in which a fatty acid ester emulsifier was used, water-in-oil emulsions were stable up to pH 9.0 (Sherman, 1955c). Above this value, inversion occurred either through interaction between ester and alkali or through soap formation between alkali and contaminating free fatty acids, which causes solubilization of the ester emulsifier.
- *Ionic conditions*. The influence of certain salts and metallic oxides used in printing fluids that affect the rheological properties of the aqueous phase and emulsions were studied by Sherman (1955b). As the conditions are not relevant to foods, ionic conditions will be dealt with later under ingredient effects.

#### 9.5.1.7. Stability of Water-in-oil Emulsions to Inversion

The effects of the concentration of the dispersed phase, emulsifier type and concentration on the inversion of water-in-oil emulsions have been described above. However, since in this chapter, it is proposed to examine the effects of a multi-ingredient system on emulsion stability, each of the following factors must be considered: emulsion formation, stability, instability, effects of shear and effects of ingredients (Becher, 1977).

#### 9.5.1.8. Emulsion Formation

The method of emulsion formation must be standardized and reproducible. The equipment used, the shear rate, the time and temperature of emulsification are the main factors in emulsion formation.

#### 9.5.1.9. Emulsion Stability

Many methods are used to measure the stability of oil-in-water emulsions. Basically, coalescence is measured by the change in emulsion droplet size with time (Halling, 1981). However, since coalescence is slow in protein-stabilized emulsions (Tornberg and Ediriweera, 1987), an accelerated method is usually used. The effects on coalescence of accelerated methods such as heating, freezing and shear stress have been measured by changes in turbidity (Pearce and Kinsella, 1978; Tornberg and Ediriweera, 1987; Melsen and Walstra, 1989). Other investigators have used measurements of particle size determined by Coulter counter (Hassander *et al.*, 1989), centrifugal photo-sedimentation (Boyd *et al.*, 1972), or an electronic imaging system (Klemaszewski *et al.*, 1989) or by other indirect methods including microwave irradiation (Petrowski, 1974) and solvent extraction of free fat (Foley *et al.*,

1971; Tornberg and Ediriweera, 1987). Phase inversion temperature (Shinoda and Saito, 1969) and emulsifying capacity (Swift *et al.*, 1961) have been used to evaluate the effects of low molecular weight and protein emulsifiers, respectively. Unfortunately, it is not possible to measure the size of the large droplets present in unhomogenized water-in-oil emulsions because the droplets coalesce very quickly. The phase inversion temperature is not a relevant test, as it may not be related directly to the stability to inversion at the emulsification temperature. Furthermore, it has been stated (Matsumoto and Sherman, 1970) that water-in-oil emulsions do not exhibit a true phase inversion temperature, unlike oil-in-water emulsions.

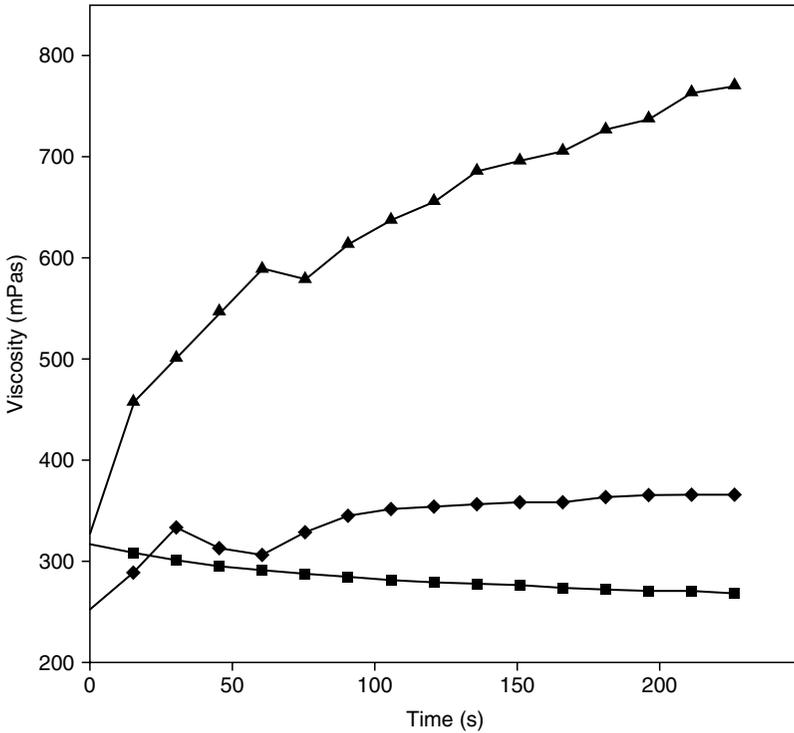
#### 9.5.1.10. Emulsion Instability

A quantitative method for assessing stability to inversion and the extent of emulsion inversion was developed by Keogh (1993). The rheological method used was based on the observation that, provided an aqueous phase with a viscosity greater than 300 mPa s at 367/s was used to prepare a 25% fat emulsion, a stable water-in-oil emulsion is slightly thixotropic, a mixed emulsion is slightly rheopectic while an incompletely inverted water-in-oil emulsion is highly rheopectic. Thus, the change in viscosity over time (240 s) at a constant shear rate (291/s) at 40°C varied from -49 mPa s for a stable emulsion to +449 mPa s for an unstable emulsion. The stability of the emulsions to inversion was also assessed visually after dispersion in cold water. The change in viscosity of a stable water-in-oil emulsion over time was negative or low (<100 mPa s), that for a mixed emulsion was intermediate (100–200 mPa s), and that for an incompletely inverted emulsion was high (>200 mPa s), as shown in Figure 9.3 (Keogh, 1993).

Measurement of the electrical conductivity of emulsions has been considered as an alternative method since oil-in-water emulsions exhibit higher conductivity than water-in-oil emulsions (Ršhl, 1972). However, this method which has been used with some success to control the level of water in butter (Prentice, 1953), has the disadvantage of being dependent on ion concentration. Therefore, certain added ions increase conductivity but might not increase stability to inversion.

#### 9.5.1.11. Effects of Shear on Emulsion Stability

There is very little published information on the effect of shear or stirring conditions on the stability of water-in-oil emulsions to inversion. It has been established (Keogh *et al.*, 1988) that water-in-oil emulsions (60g of a 7% sodium caseinate solution dispersed in 40 g milk fat) are stable to inversion only within a narrow range of throughput, refrigerant temperature and agitation rate when processed in a single-unit Votator scraped-surface



**Figure 9.3.** Viscosity of an water-in-oil (■), mixed (◆) and inverted (▲) emulsions as a function time at constant shear rate.

cooler. Until recently, such information was regarded as commercially valuable and therefore was not published. Patents usually give wide ranges of processing conditions. Frequently, the conditions given, though suitable for one scraped-surface cooling system, are misleading for another system. Although guidelines can be given, processing conditions need to be optimized for each product and processing system.

## 9.6. Effects of Ingredients on Emulsion Stability

### 9.6.1. Fat

As already outlined, the higher the viscosity of the continuous oil phase, the higher the viscosity of the emulsion. At high emulsion viscosity, the stability to coalescence is increased, and may result in increased stability

to inversion. Fats of animal, vegetable, or marine origin contain about 99% triglycerides and non-triglyceride materials can, for the most part, be ignored. The presence of small amounts of surfactants such as lecithins, monoglycerides, and cholesterol should, however, be kept in mind. The functional behavior of a fat or blend of fats will depend on:

- The melting point (determined as slip point or drop point) of the fat.
- The content of solid or crystalline triglycerides present at any temperature but especially in the range of 4–37°C.
- The crystal form which is determined by the degree of heterogeneity of the triglycerides (i.e., of the fatty acids present in the molecule). The greater the diversity of fatty acids present, the more likely the fat is to form the less stable, smaller rectangular ( $1 \times 6 \mu\text{m}$ )  $\beta'$  crystals rather than the larger (20–30  $\mu\text{m}$  diameter), more stable  $\beta$  crystals, which result in a sandy mouth-feel (Mostafa *et al.*, 1985). Sunflower oil, lard, cocoa butter and coconut or palm kernel oils tend to form  $\beta$  crystals during storage after cooling. Milk fat is naturally very heterogeneous. Palm oil, tallow and fish oils are also heterogeneous, as is lard after interesterification and partial hydrogenation. While heterogeneity can be increased by interesterification, it is usually sufficient to increase the diversity of fatty acids by adding another fat or surfactant to prevent the formation of  $\beta$  crystals. It has been shown that the presence of hexadecanoic ( $\text{C}_{16:0}$ ) (Wiedermann, 1978) or docosenoic ( $\text{C}_{22:1}$ ) acid (Hojerobá *et al.*, 1992) is particularly effective in this regard. Therefore, crystal size and crystal form are interrelated (Hoerr, 1960).

The melting point of fats used for spreads should not exceed mouth temperature (37°C) as an unpleasant waxy effect, termed palate-cling, starts to develop above this temperature.

The proportion of solid fat at any given temperature largely determines the behavior of the fat at that temperature. The content of solid fat can be measured by dilatometry, by wide-line nuclear magnetic resonance (NMR) or by pulsed NMR. The latter method is now commonly used commercially to measure the solid fat content (SFC). The behavior of fats and fat blends during processing and storage of spreads can be related to the SFC versus temperature curve. The SFC/temperature profile of a fat is related to many characteristics of the product, including general appearance, packability (in foil wrap or tub), organoleptic properties (flavor release, “coolness” and thickness), firmness/spreadability and oil exudation. The firmness or yield value of a plastic material can be measured directly using a controlled stress rheometer or indirectly using a cone penetrometer (Haighton, 1959), extruder (Prentice, 1954) or sectilometer (Knoop, 1972). Haighton (1959) developed a formula

to relate the penetration depth to the yield value, but the latter two indirect methods measure the force required to extrude and cut the sample, respectively. It is important to note that the yield value is related to, but is not equivalent to, spreadability. The reason is that when the yield value is being measured in shear flow, the velocity gradient or rate of shear is perpendicular to the direction of the stress applied. In elongational flow, the rate of shear is in the direction of the stress applied. When spreading by hand, the rate of shear lies somewhere between elongational and perpendicular flow (van Vliet, 1991). The SFC at 4 or 10°C is related to the spreadability ex-refrigerator; an SFC of not greater than 32% at 10°C should result in a butter that is spreadable at 4°C (i.e., having a maximum yield value of 1000 g/cm<sup>2</sup> at 4°C). Packability can be assessed by the yield value at 15°C. A minimum yield value in the region of 500 g/cm<sup>2</sup> is desirable for foil wrapping. The SFC at 20°C determines the tendency towards oil exudation; a value of at least 10% is essential to prevent oiling off. This value represents the level of fat crystals required to adsorb the free oil present in the product at this temperature.

Butter that is “fridge-spreadable,” but with poor stand-up qualities at 21°C, was made from combinations of mainly the low melting liquid butter-oil fraction and some of the high melting solid fraction. In addition, the use of a very high melting-point solid fraction, obtained by acetone fractionation, enabled the production of butter, which was also physically stable at 21°C. In addition to their illegal status, the fractions crystallized from solutions in acetone were also more susceptible to oxidation (Kaylegian and Lindsay, 1992). A more acceptable means of obtaining very high melting fractions is therefore required.

In low-fat spreads, margarines and shortenings, a proportion of high melting-point fats is necessary for the formation of the fat crystal network (Haighton, 1976). Aggregation of the crystals occurs to form platelets and eventually shells that surround the water droplets (Precht and Buchheim, 1980; Heertje *et al.*, 1987; 1988; Juriaanse and Heertje, 1988; Chawla *et al.*, 1990). As already mentioned, the crystals should preferably be in the small  $\beta'$  form. The absence of  $\beta'$  crystals may lead to a lack of plasticity and the possibility to inversion during the cooling process but has no direct effect on the emulsion at 40°C. However, during the preparation of emulsions, care must always be taken to melt completely crystals of fats and emulsifiers. The presence of large crystals could rupture the droplets during both stirring and cooling of the emulsion and thereby cause inversion.

### 9.6.2. Emulsifiers

Emulsifiers reduce the interfacial tension between two mutually-immiscible liquids because they have an affinity for both phases. Ford and

Furmidge (1966) indicated three essential properties of an emulsifier for concentrated water-in-oil emulsions:

- The emulsifier should reduce the interfacial tension to less than 1 mN/m.
- It should form a relatively rigid, uncharged interfacial film, through either electrostatic or hydrogen bonding between the emulsifier molecules, which will prevent the coalescence of water droplets while facilitating the coalescence of oil droplets.
- It should adsorb rapidly at the oil/water interface.

Sherman (1973) suggested that a strong interfacial film of emulsifier is also necessary to prevent the coalescence of water droplets in oil-continuous emulsions.

The emulsifier should always be more soluble in the continuous than in the dispersed phase (Bancroft, 1913). Solubility is related to polarity, which has been rated on a hydrophilic to hydrophobic scale of 20 to 1 by (Griffin, 1949, 1954). Ford and Furmidge (1966) noted that water-in-oil emulsifiers have a hydrophilic:lipophilic ratio (the so-called HLB) of 3.5 to 6.0 but interfacial viscoelasticity was not quantified. The emulsifiers used were fatty acid monoesters of either sorbitol or glycerol. Later, it was shown empirically that monoglycerides with a HLB value in the region of 3–4 and a degree of unsaturation corresponding to an iodine number of 40–55 give good stability in water-in-oil emulsions (de Feijter and Benjamins, 1978). The properties of a range of monoglyceride emulsifiers of different iodine number, fatty acid profile, HLB and slip point are summarized in Table 9.1. Monoglycerides with an iodine number of 40–55 and a melting point in the region of 55°C were found to be optimal (Grindsted, 1988) because this level of unsaturation conferred an ideal level of flexibility/rigidity on the molecule (Garti and Remon, 1984). Variations in fatty acid composition have only a slight effect on HLB but quite a marked effect on emulsion stability (Quest International, 1988). In water-in-oil emulsions, there is a positive correlation between emulsion stability and fatty acid chain length and a negative correlation with the dielectric constant of the emulsifier (Goubran and Garti, 1988). Increasing molecular weight and decreasing dielectric constant indicate greater hydrophobicity, which leads to greater impregnation of the interface and to a more stable emulsion.

### 9.6.3. Proteins

Proteins can affect the stability of emulsions by electrostatic, steric or rheological means, as already described. The mechanisms involved are highly complex, interactive and, as a result, are very difficult to quantify.

**Table 9.1.** Properties of partially hydrogenated monoglyceride emulsifiers

Commercial name <sup>a</sup>	Iodine number	Saturated fatty acids (% w/w)	Unsaturated fatty acids (% w/w)	HLB	Slip Point (°C)	Source
Dimodan S	40–50	50.4	47.7	4.3	61	Lard
Dimodan OT	55	22.2	76.8	4.3	55	Tallow olein
Dimodan CP	80	28.9	70.3	4.3	48	Vegetable oils
Dimodan LS	105	–	–	4.3	44	Sunflower oil

HLB = hydrophilic-lipophilic balance.

<sup>a</sup> Grinsted Products A/S, Brabrand, data sheets.

However, valuable insights into their mode of action have been obtained (Dalglish, 1989; Leman and Kinsella, 1989).

In order to adsorb at the interface and be surface-active, proteins must be flexible and amphipathic (i.e., have some affinity for both phases). The hydrophobic side-chains will adsorb on the oil side of the interface while the hydrophilic side-chains will interact with the aqueous phase. Three aspects of the side-chains of a protein are important:

- Content, location and grouping of the hydrophilic and hydrophobic amino acid residues.
- Surface polarity or charge that is relatively more important for emulsions than the overall charge.
- Protein structure and ability to unfold that are determined by the amino acid sequence.

Proteins are dynamic molecules with respect to structure. The preferred “folded” structure for a given set of environmental conditions is that which has the minimum free energy. The driving force to assume a given folded structure is a thermodynamic force. In aqueous systems, the hydrophobic side-chains will endeavour to orient away from the surrounding water and towards the core of the molecule. However, for high surface activity, it is essential that the protein molecule should unfold and orient its hydrophobic side-chains towards the oil phase. A lack of hydrophilic residues usually does not restrict protein functionality at interfaces. Thus, flexible proteins can create a highly hydrated, mobile layer to stabilize an emulsion particle.

One way of increasing the flexibility of a protein is to denature it (e.g., by heating or treatment with urea). Denaturation will also occur after adsorption of the protein at the interface (Graham and Phillips, 1979), a process that may be slow. The properties of an emulsion are, therefore, time-dependent.

The third mechanism by which proteins affect the stability of emulsions is rheological. This mechanism derives fundamentally from electrostatic and steric effects. The importance of viscosity has been described earlier. The viscosity of a caseinate solution is, *inter alia*, an indicator of the degree of bound water absorbed by the hydrophilic groups, as well as the water trapped inside the molecular aggregates (Korolczuk, 1982). The viscosity parameters ( $K$ , apparent viscosity at zero shear stress;  $n$ , the power law factor and  $\sigma_y$ , the yield stress) of sodium caseinate have been studied and found to be affected by concentration (Hermansson, 1975), precipitation and solution pH of caseinate (Hayes and Muller, 1961; Korolczuk, 1982), denaturation (Hayes and Muller, 1961; Canton and Mulvihill, 1982), sodium chloride (Hermansson, 1975; Creamer, 1985), calcium chloride (Hayes and Muller, 1961) and temperature (Korolczuk, 1982).

#### 9.6.4. Hydrocolloid Stabilizers

From a rheological and stability standpoint, hydrocolloid stabilizers may be used to increase the viscosity of the aqueous phase of a low-fat spread by binding water. Gelatin is of special interest, as it is a protein. However, the lack of hydrophobicity of gelatin gives it a low rating in terms of interfacial activity and consequent emulsion stability (Chesworth *et al.*, 1985). It has been shown that caseinate displaces gelatin at the oil/water interface (Dickinson *et al.*, 1985). At high ratios of caseinate:gelatin, gelatin is desorbed entirely and is found only in the aqueous phase (Musselwhite, 1966). Thus, gelatin has no electrostatic or steric role at the interface in a water-in-oil emulsion but has a role in increasing viscosity through water binding.

Other hydrocolloids that increase the viscosity of aqueous systems are also potentially useful. These include carrageenans and starch.  $\kappa$ -Carrageenan is an anionic polysaccharide, which reacts with positive sites on  $\kappa$ -caseinate (Snoeren *et al.*, 1976). This interaction can occur even when the pH of the solution is above the isoelectric point of the protein (Bettelheim *et al.*, 1966) and has been attributed to the uneven distribution of charged residues on the protein. The interaction seems to be electrostatic because it is eliminated by chemical modification of the caseinate to remove all positive sites (Day *et al.*, 1970). The maximum effect of  $\kappa$ -carrageenan occurs at lower ratios. The ratio of  $\kappa$ -carrageenan to caseinate should not exceed 1:4 as some precipitation of the aggregates occurs (Elfak *et al.*, 1979). Interaction between other carrageenans ( $\lambda$  and  $\iota$ ) and  $\alpha_s$ -( $\alpha_{s1}$ -,  $\alpha_{s2}$ -) and  $\beta$ -caseins *via*  $\text{Ca}^{2+}$  bridging also seems to occur, since these proteins bind carrageenans in the presence of  $\text{Ca}^{2+}$  (Hansen, 1968), and the casein-carrageenan interaction is minimal in the absence of  $\text{Ca}^{2+}$  (Lin and Hansen, 1970).

Starch is also of interest because it is reported to increase the viscosity of caseinate solutions in a synergistic manner, especially at caseinate concentrations  $> 10\%$  (Platt, 1988). The increase in viscosity was thought not to be due to any chemical interaction (Jones and Wilson, 1976) but rather to the increase in the swelling volume of the starch (Lelièvre and Husbands, 1989).

#### 9.6.5. Sodium Chloride

The effect of  $\text{Na}^+$  on the stability of water-in-oil emulsions is exercised mainly through its influence on sodium caseinate. It has been shown that as the surface concentration of casein on oil droplets is increased, the oil-in-water emulsion becomes less susceptible to flocculation/coalescence in the presence of electrolyte. Added NaCl broadens the droplet size distribution at a low casein content (0.25%) but causes this effect at a high casein content (0.5%) only when  $\text{CaCl}_2$  is added (Dickinson *et al.*, 1984).

#### 9.6.6. Disodium Phosphate and Trisodium Citrate

These salts have little effect on the water-binding capacity and viscosity of caseinate. Their main effect is to bind any residual calcium in the system (Vakaleris and Sabharwal, 1972).

#### 9.6.7. pH

The effects of pH on caseinate have been discussed above.

#### 9.6.8. Interactions of Ingredients

The interactive behaviour of proteins, such as sodium caseinate, and small molecule surfactants, such as monoglycerides, is very important for emulsion stability. The competitive displacement behavior of some food proteins, including casein and its fractions, has been described above. Caseinates and monoglycerides adsorb competitively at the oil-water interface. The main difference is that monoglycerides, being much smaller (mol wt.,  $\sim 380$  Da) adsorb more quickly at the interface. Information has been generated, mainly through studies on interfacial tension and more recently using confocal laser scanning microscopy.

The ability of starch, specifically amylose, to complex with l-monoglycerides may modify the activity of the monoglyceride in some way. On a weight basis, the maximum complexing ability was found with the monoglyceride of tetradecanoic acid while on a molar basis, maximum complexation occurred with the monoglyceride of octadecanoic acid. For monoglycerides derived from unhydrogenated soya oil (55%  $\text{C}_{18:3}$ ) and

hydrogenated soya oil (85% $C_{18:0}$ ) the complexing indices were 28 and 87%, respectively (Krog, 1971). Takeo and Kuge (1969) showed that the inner diameter of an amylose helix can vary from 4.5 to 6.0Å. The inner surface of the helix is lipophilic in character, but the diameter determines the size of monoglyceride molecule, which can enter. It was shown by Krog (1971) that molecules with a large polar group are poor amylose-complexing agents while molecules that have a straight hydrocarbon chain and a small polar group, such as saturated monoglycerides, are very effective complexing agents.

**9.7. Interactions of Ingredients in Low-Fat Spreads**

In low-fat stirred emulsions, the composition and rheological properties of the aqueous phase were shown to affect the stability of the emulsion to inversion (Keogh, 1993). The principal effects and interactions of the various ingredients were complex, but of very practical and commercial significance. The levels of each ingredient chosen for study are indicated in Table 9.2. When the effect of constant shear rate over time on emulsion viscosity is measured, the effect due to droplet size should also be considered. It was shown (Sherman, 1983) that viscosity increases as mean droplet size decreases but the effect is small for droplets of mean diameter >3.3 μm. The effect of droplet size, therefore, is significant in homogenized emulsions but can be ignored in stirred emulsions in which the mean droplet size is much larger.

The effect of sodium caseinate, alone (Hermansson, 1975), and with starch (Platt, 1988) or NaCl (Hayes and Muller, 1961; Hermansson, 1975), on the viscosity of an aqueous system is well documented in the literature but

**Table 9.2.** Ingredients and levels studied in water-in-oil emulsions (Keogh, 1993)

Ingredient	Ingredient (%)	
Emulsifier <sup>a</sup>	0.5	0.7
Na caseinate <sup>b</sup>	12.0	13.0
Starch	1.33	1.67
NaCl	0.67	1.33
Na <sub>2</sub> HPO <sub>4</sub> + Na <sub>3</sub> citrate	0.33 + 0.37	0.66 + 0.74
pH	5.38 – 5.69	6.60 – 6.85

<sup>a</sup> Percentage in emulsion.

<sup>b</sup> Percentage in aqueous phase.

**Table 9.3.** Main effects of the ingredients on the viscosity parameters of the aqueous phase of emulsions (Keogh, 1993)<sup>a</sup>

Ingredient	Aqueous phase viscosity parameters and percentage variance											
	K (mPas)					s.s. %/						
	Low	High	Significance	Total s.s. <sup>b</sup>	Low	High	Significance	s.s. %/Total s.s. <sup>b</sup>	n	High	Significance	
Emulsifier	–	–	NA	–	–	–	NA	–	–	–	NA	–
Na caseinate	1370	3180	***	27.7	0.85	0.80	***	–	–	–	***	13.0
Starch	1780	2770	***	8.3	0.84	0.81	***	–	–	–	***	5.3
NaCl	2020	2530	***	2.3	0.83	0.82	NS	–	–	–	NS	–
Na <sub>2</sub> HPO <sub>4</sub> + Na <sub>3</sub> citrate	2440	2110	NS	–	0.81	0.83	NS	–	–	–	NS	–
pH	3280	1270	***	34.2	0.76	0.88	***	–	–	–	***	63.3
	LSD <sub>0.001</sub> = 434					LSD <sub>0.001</sub> = 0.023						

<sup>a</sup> K is the apparent viscosity at zero shear stress and n is the power law factor

<sup>b</sup> Sum of squares (ss)/total sum of squares expressed as a percentage. NA = not available;

NS = not significant; LSD<sub>0.001</sub> = least significant difference (standard error of difference × Student's test

value) at the 0.001 level; \*\*\* = significant at the 0.001 level

the effect of phosphate + citrate (Vakaleris and Sabharwal, 1972) and pH is less clear (Hayes and Muller, 1961; Korolczuk, 1982; Creamer, 1985). The significance of these ingredients for the stability of water-in-oil emulsions to inversion has not been published.

The addition of NaCl to the aqueous phase is mainly for organoleptic reasons but its effect on caseinate must not be overlooked. Increasing the concentration of NaCl increases the ionic strength without the complications of the strong binding phenomena found with divalent cations. Large changes in the concentration of the univalent cations and their associated anion have a number of important effects on the viscosity of the aqueous phase (Table 9.3) and the stability of the emulsion. The observed small decrease in pH and the increased viscosity of the aqueous system may be related directly.

The probability exists that swelling or partial unfolding of caseinate aggregates occurs at high NaCl concentrations, thus increasing the voluminosity of the protein and hence the viscosity of the system. The associated decrease in pH at high NaCl concentrations results from the groups exposed during swelling. The steric effects of these exposed side-chains may also increase pseudoplasticity, as well as viscosity. Increasing the concentration of NaCl from 1.33 to 2%, increased pseudoplasticity. Some binding of the residual calcium by the phosphate and citrate was shown to prevent the increase in the viscosity of the aqueous phase.

Lowering the pH to 5.4 reduces the negative charge on the protein, which results in a large increase in viscosity due to partial aggregation. According to Creamer (1985), further acidification and dissociation of calcium from casein micelles results in precipitation, dehydration and reduced viscosity below pH 5.3. Similar behavior was observed (Keogh, 1993) in low-fat emulsions stabilized by sodium caseinate, although colloidal calcium phosphate is not responsible for this phenomenon, since it has already been largely solubilized by acidification and eliminated during the manufacture of casein.

Emulsions are complex systems containing many ingredients, which interact affecting the stability and rheology of the emulsion system. Many of these interactions and effects have been studied in detail by Keogh (1993).

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# Significance of Milk Fat in Cream Products

W. Hoffmann and W. Buchheim

## 10.1. Introduction

Cream is a fluid milk product, comparatively rich in fat, in the form of an emulsion of fat-in-skimmed milk, obtained by physical separation from milk (Codex Alimentarius Commission, 2003). This simple definition does not reflect that the word “cream” has for a long time been considered a premium product or a value-enhancing ingredient in milk products and other foods. The special “creaminess” results from the fine dispersion of the fat globules in the hydrophilic phase and depends strongly on the fat content. In separated cream, the diameter of fat globules ranges from *ca.* 1 to 8  $\mu\text{m}$ . During further processing to the different cream products, this typical oil-in-water (o/w) emulsion is modified or even converted into another physical state. Modification can be achieved by homogenization, which markedly reduces the average fat globule size and improves creaminess. On the other hand, mechanical treatment of chilled cream causes destabilization (i.e., coalescence of the fat globules). This treatment and the concurrent entrapment of air are essential for whipping cream into a stable foam.

The fat content of cream products varies from about 10–50%. Products with a low, internationally not-yet standardized, fat content are “coffee cream” ( $\geq 10\%$  fat, Germany), “half-and-half cream” ( $\geq 10.5\%$  fat, USA), “half cream” ( $\geq 12\%$  fat, UK) or “light cream” ( $\geq 12\%$  fat, France). Traditional whipping cream has 30 to 40% fat, whereas double cream contains about 50% fat. Creams of high fat content are also essential ingredients in dairy or non-dairy products such as some fresh cheese varieties or

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.* Springer, New York, 2006.

cream liqueurs. Butter is manufactured from cream (30–80% fat) by phase inversion. Reviews on cream, cream processing and cream products have been published by Towler (1994), Early (1998), Kessler (2002) and Hoffmann (2003). Two IDF Bulletins (IDF 1992, 1996) deal with pasteurized and UHT creams.

In summary, the significance of milk fat in the different cream products is based on fat content, fat distribution, the physical state of the fat, and last but not least, the chemical, physical, and sensory properties of the non-fat ingredients. In the following, interactions between these factors are described for the most important cream products.

## 10.2. Coffee Cream

In many countries, coffee cream is a popular long-life product, which competes with evaporated milk, whole milk, and liquid or dried coffee whiteners. In this section, “coffee cream” does not mean a national statutory term, but an appropriate description of a functional property. Such creams usually contain 10 or 12% fat, less frequently 15, 18, or even 20%. Traditionally, coffee creams are sterilized in bottles or cans. During the last 20 years, continuous-flow sterilization in a UHT plant, followed by aseptic packaging, has replaced the former process to a large extent. The products need good stability both during storage and in hot coffee beverages, as well as acceptable sensory properties.

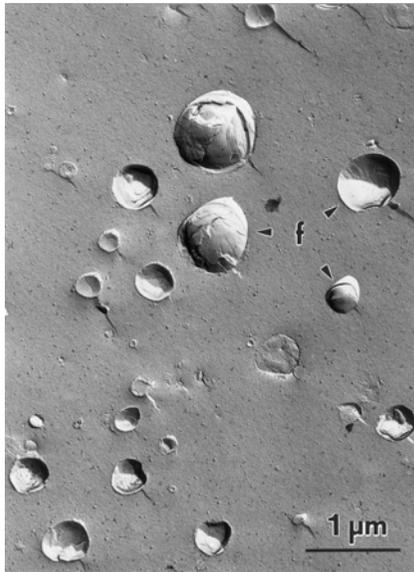
A shelf-life of several months at ambient temperature requires particularly low creaming and sedimentation in the package, which is facilitated by a low fat content (10 or 12%) and optimized processing conditions, mainly heat treatment and homogenization. Coffee creams with  $\geq 15\%$  fat need chilled storage to prevent irreversible creaming.

The different creams may contain stabilizing salts, which can be added as an aqueous solution after standardization and preheating (high-temperature pasteurization at 90–95°C). They raise the pH and/or complex  $\text{Ca}^{2+}$ , resulting in reduced aggregation of casein micelles during sterilization and in hot coffee beverages. With an increasing degree of condensation (chain length), phosphates have a reduced buffering capacity, and increased ion exchange ability. Trisodium citrate has both buffering and sequestering properties and is used also. Whereas phosphates and citrates are essential additives in traditionally-sterilized cream, high-quality flow-sterilized creams (containing 10 or 12% fat) may be produced without additives.

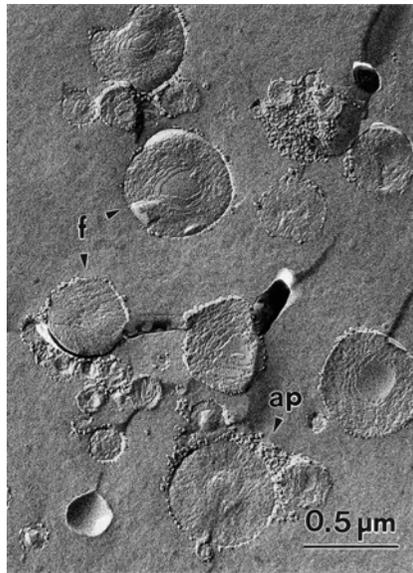
Homogenization of cream results inevitably in the formation of a secondary fat globule membrane, consisting predominantly of micellar casein and (denatured) whey proteins (Walstra *et al.*, 1999). To obtain desirable product properties, the formation of larger, thermally induced

protein aggregates, and, particularly, fat/protein complexes must be avoided (Buchheim *et al.*, 1986). The number and dimensions of the particles are influenced more by temperature than by heating time during flow sterilization. In general, such adverse structures are reduced by flow sterilization at  $\leq 130^{\circ}\text{C}$  rather than at UHT temperatures ( $\geq 135^{\circ}\text{C}$ ). Frequently, a (second) two-stage homogenization step is carried out after heating in order to disrupt heat-induced fat/protein aggregates. Sensory effects of a lower heating temperature and a prolonged heating time ( $\geq 1$  min, necessary for safe sterilization) are a more pronounced cooked flavor and a more brownish color of the resulting cream. These effects are partially masked after addition to the coffee beverage and are, therefore, of minor relevance.

Physical properties of flow-sterilized cream can be controlled most effectively by homogenizing conditions. Usually, one homogenizer is integrated up-stream (i.e., before flow sterilization) and one down-stream. Both homogenizers often operate at a total pressure of about 20 MPa at  $70^{\circ}\text{C}$ . An optimal coffee cream should have a narrow fat globule size distribution with a volume-mean diameter preferably between 0.4 and  $0.6\ \mu\text{m}$  and a very low degree of aggregation (Figure 10.1). This results in low viscosity, high whitening power, slow creaming and high “coffee stability” (i.e., resistance to feathering in hot coffee beverages).



**Figure 10.1.** Electron micrograph of flow-sterilized coffee cream; f: homogenized fat globules.



**Figure 10.2.** Electron micrograph of floccules in a coffee cream after feathering in a hot coffee solution (enlarged compared to Figure 10.1.1); f: fat globules; ap: aggregated protein.

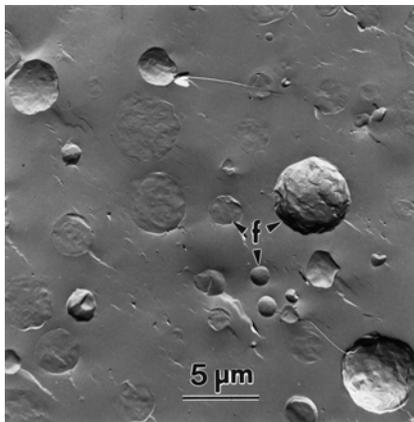
The coffee stability is particularly important for the quality of the product. It is also affected by the coffee brand and concentration, minerals present in the water, brewing conditions, and by temperature (Kessler, 2002). Typical coffee beverages have a pH of about 5.0, which is near the isoelectric range of casein. High temperature ( $\geq 70^{\circ}\text{C}$ ), low water hardness, low pH or a high concentration of sulphates accelerate protein coagulation, and hence, fat/protein aggregation. Therefore, it must be ensured that this feathering remains invisible to the naked eye (Buchheim *et al.*, 1986; Hoffmann *et al.*, 1996) (Figure 10.2). The probability of feathering increases with the fat content of the cream. Apart from feathering, floccules of condensed cream may float on the coffee surface when using cream from small polystyrene (PS) portion packs. Considerable loss of water (about 10–15% of cream weight during 4 months) occurs with deep-drawn PS containers, which facilitates the formation of such floccules in coffee.

### 10.3. Whipping Cream

Whereas the processing of long-life coffee cream is characterized by severe homogenization and heat treatment, traditionally pasteurized (at *ca.*  $85^{\circ}\text{C}$ ) whipping cream is produced carefully with little thermal and mechanical

input (Figure 10.3). However, the demand for a longer shelf-life has led to a subsequent high-temperature pasteurization ( $\geq 110^{\circ}\text{C}$ ) or even UHT heating and additional low-pressure homogenization. The higher thermal load results in more or less cooked flavor. Although the prolonged shelf-life requires efficient steps against irreversible creaming (plug formation), adequate storage stability of the double-pasteurized cream containing 30–35% fat can, however, be achieved without homogenization. The addition of hydrocolloids (particularly fractions of carrageenan), milk constituents (whey proteins and high-melting fat fractions, see Precht *et al.*, 1988) and even synthetic emulsifiers (if legally permitted) can slow down creaming during 3 weeks at  $\leq 10^{\circ}\text{C}$ . The aim of UHT treatment is to produce sterile cream with a shelf-life up to 3 months at about  $20^{\circ}\text{C}$ . Usually, indirect heating at  $\geq 135^{\circ}\text{C}$  with a short holding time of a few seconds is applied in order to limit the thermally induced physical, chemical and sensory changes. Unchilled storage without serious creaming requires the use of stabilizers (hydrocolloids and/or synthetic emulsifiers), a fat content near the lower limit of 30%, but also a slight reduction in the size of the original fat globules. The homogenization effect must be moderate in order to retain acceptable whipping properties. A compromise between long storage stability and adequate functional attributes is needed. Homogenization and subsequent UHT heating would, however, cause an increase in the content of free fat. Therefore, a down-stream two-stage homogenization of cream at a total pressure of not more than 4 MPa is used frequently.

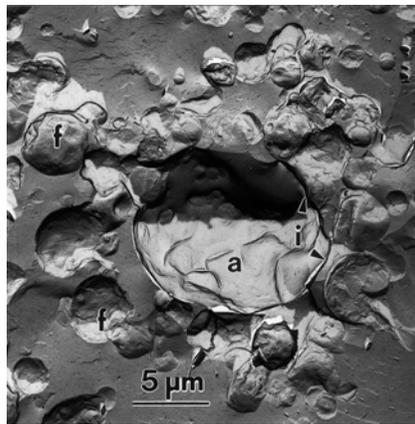
The whipping of pasteurized cream containing  $\geq 30\%$  fat is possible only after adequate cooling since the transformation of the original o/w emulsion into a stable foam requires that part of the fat is solid. The initial stage of



**Figure 10.3.** Electron micrograph of fluid whipping cream; f: fat globules.

whipping involves stabilization of the trapped air bubbles by a temporary interfacial film of soluble whey proteins and  $\beta$ -casein. On mechanical treatment, fat globules increasingly loose at least segments of their natural membrane, thereby exposing strongly hydrophobic surface areas of pure fat. Subsequently, these partly destabilized fat globules adsorb at the air/serum interface of the air bubbles (Figure 10.4). The leakage of liquid fat from mechanically stressed and deformed fat globules supports globule agglomeration and partial coalescence. These agglomerates also interact with the air bubbles and may form bridges between them. The above, highly dynamic and concurrent processes also apply on the whole to low-homogenized UHT whipping cream containing about 30% fat. Details of the interactions and processes during whipping are described by Anderson *et al.* (1987), Anderson and Brooker (1988), Buchheim (1991), Buchheim and Dejmek (1997) and Smith *et al.* (1999).

The whipping properties of creams are assessed by whipping time, increase in volume (expressed as overrun), foam firmness and by subsequent serum leakage. Comparative studies require standardized temperature, whipping and other handling conditions. Most test whipping devices are modifications of that described originally in 1937 by Mohr and Baur (see Hoffmann, 2003). Whipping of a typical cream increases the volume by 80–125% by inclusion of ambient air. UHT-treated creams can also be aerated by means of suitable propellants (e.g.,  $N_2O$ ), resulting in a volume increase in the range of about 300–500%. These convenience products are filled into sterilized aluminium or tin-plate cans. Compared with regular whipped cream, clearly more fat globules adsorb at the air interfaces, and,



**Figure 10.4.** Electron micrograph of whipped cream; a: air cell; f: fat globules; i: interfacial layer.

simultaneously, agglomeration of fat globules is reduced substantially, which results in an impaired network formation between the air bubbles. Due to the different foaming process, aerosol creams develop only soft foams with low stability (Buchheim, 1991).

Whipping cream ranks among premium food products and is consumed for its pure flavor. High-quality raw milk is essential for this product. Raw milk and separated cream must be handled carefully to minimize damage to the natural fat globule membrane. Excessive agitation and pumping should be avoided. The flow velocity should not exceed the critical shear rate, which can be calculated (Kessler, 2002). Incorporated air bubbles increase the risk of damaged fat globules or can act as centres for fat globule aggregation and subsequent coalescence. During crystallization, fat globules are most sensitive to mechanical treatment. As a result of the partial or complete loss of the protective membrane, both indigenous and bacterial lipases catalyse the hydrolysis of exposed fat to fatty acids, imparting rancid taints (Kosinski, 1996). When raw cream is homogenized without being subjected immediately to high-temperature pasteurization, indigenous milk lipoprotein lipase penetrates the secondary membrane of fat globules (which has higher interfacial tension than native membrane) and hydrolyzes triglycerides to free fatty acids within a few minutes, resulting in intense rancidity (Walstra *et al.*, 1999).

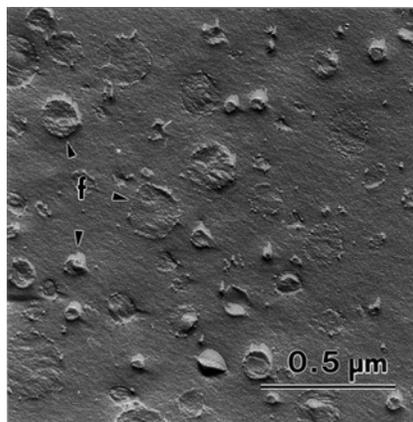
Active extracellular bacterial lipases and proteinases of *Pseudomonas* spp. and most other Gram-negative psychrotrophs may be present even in UHT cream if refrigerated raw milk had been stored for a prolonged period. They can contribute to rancid and tallowy flavors, and also to bitter cream or serious physical changes such as gelation (Castberg, 1992; Driessen and van den Berg, 1992; Houlihan, 1992; Kosinski, 1996).

Flavor defects in cream may occur not only during manufacture but also during transport or storage until the best-before date. UHT whipping cream, with its long shelf-life at ambient temperature, is particularly susceptible to off-flavors. Hence, adequate packaging materials must be chosen. Protection against oxygen and/or light is most important as they may induce oxidation of unsaturated fatty acids, leading to flavor deterioration. Paper cartons with a coating of polyethylene and an aluminium foil laminated to the inner carton layer are often used. Appropriate filling conditions should also be selected to minimize the oxygen content of the package and the cream. However, a certain level of residual oxygen may be beneficial as the UHT process exposes free sulphhydryl groups and causes the release of hydrogen sulphide from  $\beta$ -lactoglobulin, thus creating the typical cooked flavor. During storage, oxidation of these groups occurs and most of the cooked flavor disappears. A balanced antioxidative/oxidative action of sulphur groups and oxygen will probably help to ensure cream products of good taste and odor (Eyer *et al.*, 1996).

An important factor for the physical stability of cream is the temperature of the cream during transport and storage. Even a brief warming to  $\geq 30^{\circ}\text{C}$  supports creaming during subsequent storage at  $20^{\circ}\text{C}$  and may lead to a distinct thickening after cooling before whipping. Continuous cooling during the whole shelf-life delays creaming, avoids destabilization phenomena and sensory changes, and results in increased foam volume, but a longer whipping time (Hoffmann, 1999).

#### 10.4. Cream Liqueurs

Cream liqueurs combine the flavor of alcoholic drinks with the texture of cream in products with a shelf-life of several years at ambient temperature. During that period, the liqueur must be resistant to both microbiological and physical changes. The microbiological safety is guaranteed by a sufficient concentration of alcohol ( $\geq 14\%$ ) together with a high sugar content (about  $19\%$ ). Avoiding serious phase separation is the more demanding challenge. This can be achieved by optimal composition and processing. The addition of sodium caseinate (*ca.*  $3\%$ ), trisodium citrate (*ca.*  $0.2\%$ ) and possibly low molecular-weight emulsifiers like monoglycerides (*ca.*  $0.1\%$ ) stabilizes the *o/w* emulsion of the added cream (e.g.,  $16\%$  of  $48\%$  fat cream) in the liqueur. In the final product, more than  $98\%$  of the fat globules should have a diameter  $< 0.8\ \mu\text{m}$ , resulting in clearly enhanced viscosity, creaminess and whitening power (Banks and Muir, 1988). The typical volume-mean diameter of about  $0.2\ \mu\text{m}$  is, by far, the smallest of all dairy

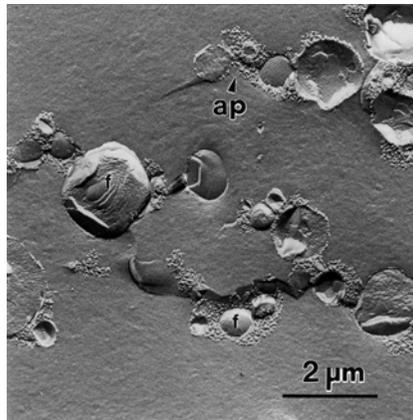


**Figure 10.5.** Electron micrograph of cream liqueur; f: fat globules.

products (Buchheim and Dejmek, 1997) (Figure 10.5). This is achieved by multiple high-pressure homogenization. Banks and Muir (1988) recommended the addition of alcohol after homogenization resulting in the formation of fewer large fat globules. Compared to unhomogenized cream, the total fat surface area increases by a factor of about 20 (up to *ca.* 40 m<sup>2</sup>/g fat) in cream liqueurs. Therefore, sodium caseinate is dissolved first in hot water before adding the cream, sugar, citrate and a complementary emulsifier (if necessary). No other protein than sodium caseinate is able to provide the required long-term emulsion stability. O'Kennedy *et al.* (2001) isolated special fractions of commercial sodium caseinate (soluble in high concentrations of ethanol), which keep a constant viscosity of cream liqueur during storage. Trisodium citrate, a useful stabilizer for several dairy products, such as evaporated milk or sterilized coffee cream, complexes the Ca<sup>2+</sup> and concurrently increases the pH. In cream liqueur, trisodium citrate prevents the interaction between sodium caseinate and available calcium. Otherwise, gelation and syneresis during storage would occur. If a cream liqueur with a substantially higher alcohol content than 14% is produced (e.g., 19%), a second addition of alcohol after homogenization of cream (and other ingredients) is required in order to produce a stable emulsion. The manufacture of cream liqueurs ends with filling into brown glass bottles to prevent light-induced off-flavor. Very occasionally, during long-term storage, the formation of a non-redispersible cream or fat plug in the neck of the bottle may occur (Dickinson *et al.*, 1989). The fatty solid-like cohesive structure of this plug points to unfavorable ambient temperatures, possibly accompanied by excessive mechanical agitation. The formation of neck-plug may be similar in origin to the thickening of whipping cream after warming for a short period ( $\geq 30^{\circ}\text{C}$ ) and subsequent cooling.

## 10.5. Cultured Cream

Cultured or sour(ed) creams find various applications as valuable additives for dishes and in refining sauces and dressings. They are manufactured in many countries and their fat content generally ranges from 10 to more than 40%. The production is largely equivalent to that of other fermented milk products (Puhan, 1988). It starts with standardization of the fat content and may include the addition of skim milk concentrate or skim milk powder, milk protein and hydrocolloids (e.g., gelatin or starch), if legally permitted. These ingredients improve texture and prevent syneresis of the final product. Adequate processing conditions and a higher fat content reduce the need for supplementation. The homogenization pressure required for cream decreases with increasing fat content. Homogenization after high-temperature



**Figure 10.6.** Electron micrograph of cultured cream (10% fat); f: fat globules; ap: aggregated protein.

pasteurization results in better consistency compared to up-stream treatment. The fat globules participate directly in the following fermentation process and are integrated into the developed network (Buchheim and Dejmek, 1997) (Figure 10.6). Normally, the use of mesophilic lactic acid bacteria results in a long fermentation time (14–24 h). Chemical acidification (e.g., by glucono- $\delta$ -lactone, lactic acid) is uncommon. Typical cultured cream products should be uniform (without creaming), creamy and viscous with a slightly acidic, mild “cheesy” or “buttery” flavor. Cultured creams may also develop a nearly plastic consistency as a result of modified composition and/or appropriate production and may be used as low-fat spreads (o/w type).

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# Significance of Milk Fat in Cheese

T.P. Guinee and P.L.H. McSweeney

## 11.1. Introduction

Fat is a major component in most cheese types, but its level and importance differ markedly with variety. Inter- and intra-variety differences in fat content are affected by a number of factors, including milk composition (particularly ratio of protein to fat), and the cheesemaking process (recipe, manufacturing procedure and technology), which control the levels of milk fat and moisture retained in the cheese curd and the moisture content of the cheese. The ratio of protein-to-fat in the cheese milk is probably the principal factor influencing fat content, as it controls the relative proportions of two of the three major compositional components in cheese, namely protein and fat; the third major component is moisture. Owing to the inverse relationship between the percentage of moisture and fat in cheese, as discussed in Section 11.2.1, differences in moisture content can lead to relatively large differences in the fat content of cheese varieties of similar fat-in dry matter (FDM) content [e.g., Comte cheese (30% fat, 33.5% moisture, 45% FDM), Danish Havarti (~26.5% fat, 43.5% moisture, 46.9% FDM), Tilsiter cheese (26.5% fat, 42.3% moisture, 46% FDM) and Coulommiers (22% fat; 53% moisture, 46.8% FDM)]. Therefore, it may be more meaningful, to express fat content as a % of dry matter (% FDM) rather than as fat on a total weight basis (% w/w).

The FDM content is between 42–56% for most of the rennet-curd cheese varieties (e.g., Cheddar, Gouda, Blue, Brie), but varies from ~33% in Grana Padano and low-moisture part-skim Mozzarella to ~70% in Cambozola (see USDA, 1976; Holland *et al.*, 1991; Robinson, 1995; Kosikowski

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

and Mistry, 1997); the FDM of the reduced-fat versions of these cheeses is, of course, lower [e.g., ~52 and 31% for full-fat (33%, w/w, fat) and half-fat (17%, w/w, fat) Cheddar cheese, respectively]. Usually, the FDM of fresh acid-curd cheeses is relatively low (< 40% FDM), apart from Double Cream cheese (~73% FDM) and Mascarpone (~89% FDM) (Schulz-Collins and Senge, 2004).

The level of fat influences several aspects of cheese, including composition, biochemistry, microstructure, yield, rheological and textural properties, and cooking properties (Bryant *et al.*, 1995; IDF, 1991; Mistry and Anderson, 1993; Tunick *et al.*, 1993a,b; Merrill *et al.*, 1994; Olson and Bogenrief, 1995; Fife *et al.*, 1996; Rudan and Barbano, 1998; Rudan *et al.*, 1999; McMahan *et al.*, 1999; Guinee *et al.*, 1998, 2000a; Fenelon and Guinee, 1999, 2000; Fenelon *et al.*, 2000b; Metzger *et al.*, 2001a,b; Gwartney *et al.*, 2002; Michaelidou *et al.*, 2003a,b). Moreover, for a given fat content, the type of fat (melting point) and the state of the fat (non-globular, free fat, homogenized, globule size distribution, solid-to-liquid ratio) has a major impact on the rheology, flavor and cooking properties of cheese (Lelievre *et al.*, 1990; Metzger and Mistry, 1995; Poudaval and Mistry, 1999; Tunick *et al.*, 1993a,b; Rudan *et al.*, 1998b; Wijesundra *et al.*, 1998; Nair *et al.*, 2000; Oommen *et al.*, 2000; Guinee *et al.*, 2000a). Fat also contributes to flavor directly and indirectly *via* lipolysis (Balcao and Malcata, 1998; Gripon, 1997; Guinee and Law, 2002). Moreover, in some varieties, free fatty acids (FFAs) serve as precursors for other flavor compounds, (e.g., in Blue cheeses) in which FFAs are oxidised to methyl ketones, which in turn may be reduced to secondary alcohols (Collins *et al.*, 2004).

The increased affluence in western society has resulted in excessive calorie intake, and expert panels have recommended reduction in the intake of both total and saturated fat (see O'Brien and O'Connor, 2004). Dietary fat has been shown to be associated with an increased risk of obesity, atherosclerosis, coronary heart disease, elevated blood pressure, and tissue injury diseases associated with the oxidation of unsaturated fats (Hayes, *et al.*, 1991; Hodis *et al.*, 1991; Doyle and Pariza, 2002; McNamara, 1995; Simon *et al.*, 1996;). This has created increased consumer concern over the implications of dietary fat on health, and large increases in the supply, and demand for, low-fat foods, including cheese (Shank and Carson, 1990; Dexheimer, 1992). However, it is generally acknowledged by market experts that the consumption of low- and reduced-fat cheeses remained relatively low (e.g., 8% of total cheese in the UK) throughout the 1990s, though some current commercial information indicates large increases in the market for low-fat cheese varieties (e.g., see [www.arlafoods.com](http://www.arlafoods.com)). The relatively low consumption of reduced-fat cheese has been attributed to poor consumer perception of the products based on taste and texture (Olson and Johnson,

1990; Bullens, 1994; Anonymous, 1996). Textural defects include increased firmness, rubberiness, elasticity, hardness, dryness, and graininess. The negative flavor attributes of reduced-fat Cheddar include bitterness (Ardö and Mansson, 1990) and a low intensity of typical Cheddar cheese aroma and flavor (Banks *et al.*, 1989; Jameson, 1990). Approaches used to improve the quality of reduced-fat cheese include:

- (1) Alterations to the cheese making procedure to reduce the calcium-to-casein ratio, increase the moisture-to-protein ratio and reduce the extent of *para*-casein aggregation [e.g., by high pasteurization temperature, high pressure treatment of milk, reducing the pH at setting and whey drainage, and/or increasing gel firmness at cutting (e.g., Banks *et al.*, 1989; McGregor and White, 1990; Metzger and Mistry, 1994; 1995; Guinee *et al.*, 1998; Fenelon *et al.*, 1999; Rudan *et al.*, 1998b; Poduval and Mistry, 1999; Molina *et al.*, 2000; Nair *et al.*, 2000)]
- (2) The use of specialized starter cultures and starter culture adjuncts, and/or exogenous enzymes (e.g., Ardö *et al.*, 1989; Skeie *et al.*, 1995; Midje *et al.*, 2000; Tungjaroenchai *et al.*, 2001; Broadbent *et al.*, 2002; Fenelon *et al.*, 2002; Katsiari *et al.*, 2002)
- (3) The addition of fat mimetics to the milk (e.g., Desai and Nolting, 1995; Kucukoner and Haque, 1995; McMahon *et al.*, 1996; Fenelon and Guinee, 1997; Rudan *et al.*, 1998a; Bhaskaracharya and Shah, 2001; El-Sheikh, *et al.*, 2001).

These approaches have been reviewed extensively (Jameson, 1990; Ardö, 1997; Fenelon and Guinee, 1997; Fenelon, 2000). Various recommendations for the manufacture of reduced-fat cheeses with improved sensory and textural properties (Mistry *et al.*, 1996; Johnson *et al.*, 1998), (e.g., half-fat Cheddar prepared by: homogenization of cream used to standardize the cheese milk) (Nair *et al.*, 2000); the combined effects of increases in milk pasteurization temperature and pH at curd milling, and the use of selected starters and starter culture adjuncts (Guinee *et al.*, 1999; Fenelon *et al.*, 2002);

The focus of this chapter is on the generic effects of fat on the composition, structure, yield, flavor, rheology and functionality of hard and semi-hard cheeses and pasteurized processed cheese products.

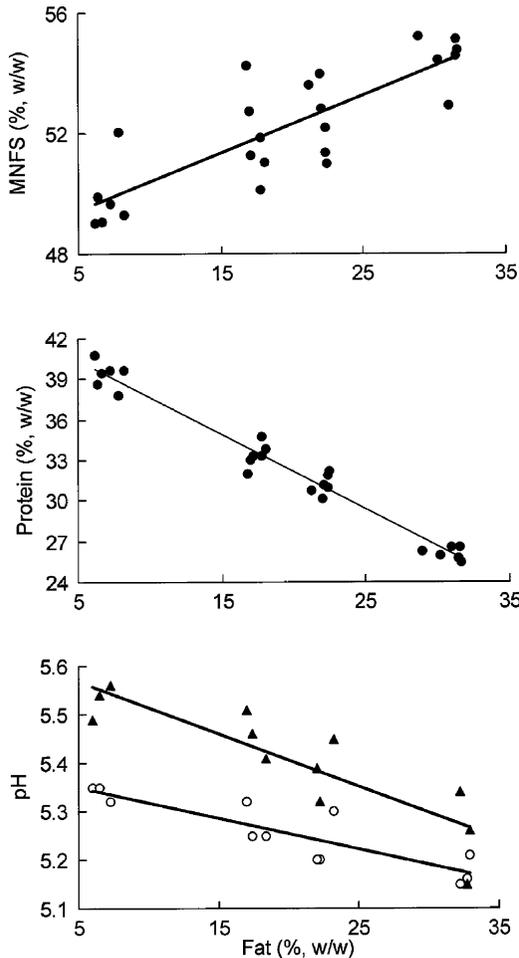
## 11.2. Effect of Fat on Cheese Composition

### 11.2.1. Fat Content

Fat is a major compositional component of most cheese varieties and major changes in its level result in concomitant changes in the levels of

moisture and protein, and in cheese yield (Drake *et al.*, 1995a, b; Fenelon and Guinee, 1999; Melilli *et al.*, 2002).

Numerous workers have investigated the effects of the fat content of milk on the composition of several cheese types, including Cheddar and Mozzarella (Gilles and Lawrence, 1985; Tunick *et al.*, 1991, 1993a; Bryant *et al.*, 1995; Nauth and Ruffie, 1995; Drake *et al.*, 1996b; Fenelon and Guinee, 1999; Rudan *et al.*, 1999). In studies where cheese making conditions are held constant, reduction of the fat content of cheese is paralleled by



**Figure 11.1.** Effect of fat content on the composition of Cheddar cheese; pH is shown for the 60 (○) and 180 (▲)-day old cheeses (redrawn from data of Fenelon and Guinee, 1999; Guinee *et al.*, 2000a).

**Table 11.1.** Gross Composition of Some Cheese Varieties

Cheese type	Fat (%, w/w)	Moisture (%, w/w)	Protein (%, w/w)	MNFS <sup>a</sup> (%, w/w)	Calcium (mg/100g)	Phosphorus (mg/100g)	pH
Cheddar <sup>b</sup>							
low-fat	7.2	46.1	38.5	49.6	1097	839	5.52
half-fat	17.2	43.0	33.3	51.9	937	680	5.45
reduced-fat	21.9	40.9	31.0	52.4	872	639	5.37
full-fat	30.4	37.8	26.4	57.0	742	533	5.25
Feta <sup>c</sup>							
	21.9	56.4	15.9	72.2	n.a. <sup>a</sup>	n.a.	4.57
	6.5	66.8	20.1	71.4	n.a.	n.a.	4.68
Kefalograviera-type <sup>d</sup>							
	9.8	48.4	33.4	53.6	n.a.	n.a.	5.40
	30.6	37.8	26.1	54.6	n.a.	n.a.	5.49
Mozzarella <sup>e</sup>							
reduced-fat	12.3	48.5	32.8	55.3	n.a.	n.a.	n.a.
reference	21.2	47.0	25.5	59.6	n.a.	n.a.	n.a.
Mozzarella <sup>f</sup>							
low-fat	2.2	51.2	24.6	64.5	n.a.	n.a.	n.a.
low-fat	5.0	62.5	30.4	64.5	n.a.	n.a.	n.a.
part-skim	19.3	63.6	30.1	65.0	n.a.	n.a.	n.a.
Mozzarella <sup>g</sup>							
low-fat	9.9	54.0	n.a.	60.0	n.a.	n.a.	n.a.
high-fat	24.4	48.5	n.a.	64.5	n.a.	n.a.	n.a.

<sup>a</sup> Abbreviations: MNFS, moisture in non-fat cheese substances; n.a., not available

<sup>b</sup> From Guinee *et al.* (2000a); pH measured at 120 d.

<sup>c</sup> From Michaelidou *et al.* (2003a); pH measured at 120 d.

<sup>d</sup> From Michaelidou *et al.* (2003a); pH measured at 90 d.

<sup>e</sup> From Puduval and Mistry (1999).

<sup>f</sup> From Fife *et al.* (1996).

<sup>g</sup> From Tunick *et al.* (1995).

increases in the concentrations of moisture and protein and reductions in the levels of fat-in-dry matter (FDM), moisture-in-non-fat substance (MNFS), and pH (Figure 11.1; Table 11.1). The unit changes in the latter compositional parameters on reducing the fat content in the range 33 to 6% (w/w) for Cheddar cheese were: +0.36 g moisture/g fat, +0.55 g protein/g fat, +0.05 g ash /g fat, -0.2 g MNFS/g fat, and -1.5 g FDM/g fat (Fenelon and Guinee, 1999). The increase in cheese pH as the fat content is reduced may be attributed to the concomitant decrease in the level of MNFS and, hence, lactate-to-protein ratio (Fenelon and Guinee, 2000). A similar effect was observed in cheeses with a similar level of protein but with different levels of lactic acid, as affected by altering the lactose concentration in the cheese milk (Shakeel-Ur-Rehman *et al.*, 2004).

A small increase in MNFS (2–4%, w/w) leads to a relatively large increase in free, available water, which in turn leads to increases in the activity of microorganisms and enzymes and the degree of proteolysis in cheese (Creamer, 1971; Pearce and Gilles, 1979; Lawrence and Gilles, 1980; Lawrence *et al.*, 1987). Hence, normalization of the MNFS is considered especially important to improve the quality of reduced-fat cheeses. Consequently, in commercial cheese manufacture and in many studies relating to the improvement of the quality of reduced-fat cheese, cheese making procedures are frequently altered so as to give reduced-fat cheese with a level of MNFS similar to that of the full-fat equivalent (Banks *et al.*, 1989; Ardö, 1993; Drake *et al.*, 1995b). Hence, the fat content of retail Cheddar cheese is inversely correlated with the levels of moisture and protein but it does not significantly affect the level of MNFS (Banks *et al.*, 1992; Fenelon *et al.*, 2000b).

### 11.2.2. Effect of Degree of Fat Emulsification as Influenced by Homogenization of Milk, Cream and/or Curd

It is generally accepted that homogenization of milk, or the cream used to standardize the cheese milk, at a combined first-stage and second-stage pressures of 5–20 MPa, reduces the degree of curd syneresis (Pearse and MacKinlay, 1989) and thereby increases the levels of moisture and MNFS in cheese (Table 11.2). Cheeses, for which increases in moisture or MNFS have been reported, include Cheddar of different fat content (Emmons *et al.*, 1980; Metzger and Mistry, 1994; Nair *et al.*, 2000), Edam (Amer *et al.*, 1977), Gouda (Versteeg *et al.*, 1998) and Mozzarella (Jana and Upadhyay 1991, 1992, 1993; Tunick *et al.*, 1993a; Rudan *et al.*, 1998b). A similar trend was reported for the effect of high-pressure (~100 MPa) homogenization of milk for goats' milk cheese (Guerzoni *et al.*, 1999) and Cheddar (O'Mahony, Hayes, McSweeney and Kelly, unpublished results). The extent of the increase in moisture varies depending on homogenization pressure and cheese making practices (Jana and Upadhyay 1991; 1992; Table 11.2). Oommen *et al.* (2000) reported an interactive effect between the protein content of cheese milk (as varied by ultrafiltration) and homogenization of cream (at first and second stage pressures of 6.9 and 3.5 MPa, respectively) on the levels of moisture and MNFS in Cheddar cheese, with the magnitude of the increase decreasing as the milk protein level was increased from 3.2 to 6.0% (w/w). Hence, homogenization of milk may be a convenient means to offset the reductions in moisture (~3%, w/w) and MNFS in Cheddar when the protein content of milk is increased from 3 to 5%, w/w, by ultrafiltration (Guinee *et al.*, 1996, 2004b; Oommen *et al.*, 2000). Microfluidization of milk at 7 MPa or cream at 14 or 69 MPa also increases the moisture content in Cheddar cheese (Lemay *et al.*, 1994) (Table 11.2).

**Table 11.2.** Effect of Homogenization or Microfluidization of Milk, or Cream, on the Composition of Cheese

Treatment	Treated	Cheese	Composition (% w/w)					Reference
			Fat	FDM <sup>a</sup>	Protein	Moisture	MNFS <sup>a</sup>	
Microfluidisation <sup>b</sup> pressure (MPa)		Cheddar						
0	Milk		34.4	52.9	25.5	35.0	53.4	Lemay <i>et al.</i> (1994)
7	Milk		34.0	54.5	23.0	37.6	57.0	
14	Cream		33.4	54.3	22.6	38.4	57.7	
69	Cream		33.0	36.6	22.1	39.3	58.6	
Homogenization <sup>c</sup> pressure (MPa)		Cheddar						
0	Milk		29.9	49.2	25.3	39.2	55.9	Guinee <i>et al.</i> (2000c)
30	Milk		30.6	51.2	23.9	40.2	57.9	
Homogenization <sup>d</sup> pressure (MPa)		Cheddar						
0	Cream		33.1	53.3	24.7	37.9	56.7	Nair <i>et al.</i> (2000)
7	Cream		32.9	54.1	23.9	39.2	58.4	
10.4	Cream		32.8	53.7	23.6	38.9	57.9	
13.7	Cream		32.1	52.8	23.5	39.3	57.8	
Homogenisation <sup>e</sup> pressure (MPa)		Reduced-fat Cheddar						
0	Cream		17.7	32.9	30.5	46.0	55.9	Metzger and Mistry (1995)
20.7	Cream		17.8	33.6	29.6	47.1	57.3	
Homogenization <sup>f</sup> pressure (MPa)		Half-fat Cheddar						
0	Milk		18.2	31.8	33.5	42.7	52.2	Fenclon (2000)
15	Milk		18.0	32.1	32.6	44.0	53.6	
Homogenization <sup>b</sup> pressure (MPa)		Mozzarella						

(Continued)

Table 11.2. (Continued)

Treatment	Treated	Cheese	Composition (% w/w)						Reference
			Fat	FDM <sup>a</sup>	Protein	Moisture	MNFS <sup>a</sup>		
0	Milk		25.8	47.8	21.5	46.0	62.0	Jana and Upadhyay (1991)	
4.5	Milk		26.5	54.6	19.0	51.4	69.9		
		Mozzarella							
0			25.9	48.2	n.a. <sup>a</sup>	46.3	62.4	Tunick <i>et al.</i> (1993b)	
10.3			26.9	51.7	n.a.	47.9	65.5		
17.2			27.8	52.2	n.a.	46.8	64.8		
		Reduced-fat Mozzarella							
0			11.1	23.3	n.a.	52.5	59.0	Tunick <i>et al.</i> (1993b)	
10.3			9.4	20.9	n.a.	55.1	60.8		
17.2			10.8	23.5	n.a.	23.9	60.5		

<sup>a</sup> Abbreviations: FDM, fat-in-dry matter; MNFS, moisture-in-non-fat substances; n.a., not available.

<sup>b</sup> Total pressure; first and second stage pressures not specified.

<sup>c</sup> First/second stage pressures of 25/5 MPa, respectively.

<sup>d</sup> The first and second stage pressures (MPa) for the different homogenization treatments were: 3.5/3.5, 6.9/3.5, and 10.4/3.5, respectively.

<sup>e</sup> First/second stage pressures of 17.3/3.4 MPa, respectively.

<sup>f</sup> First/second stage pressures of 10/5 MPa, respectively

The impaired syneresis in curd/cheese made from homogenized milk may be due to the increased interaction between the casein and the fat, which reduces the surface area of the casein micelles available for mutual interaction (Green *et al.*, 1983). Curd syneresis is the physical expulsion of whey, which accompanies contraction of the protein matrix. Matrix contraction may be viewed at the microstructural level as an increased aggregation and joining of adjacent casein strands into larger aggregates, both within curd particles and at curd particle junctions (Kimber *et al.*, 1974). The reduction in casein hydration and the increase in matrix contraction that parallels casein aggregation reduce the ability of the matrix to retain whey. Hence, a lower degree of casein-casein interaction in curds made from homogenized milk would lead to higher moisture content. The increase in the fineness of rennet-induced milk gels that accompanies milk homogenization (Green *et al.*, 1983) may also contribute to the higher moisture content of homogenized milk cheeses; a finer gel has lower porosity, a factor that would be expected to impede moisture expulsion.

## 11.3. Contribution of Fat to the Microstructure of Cheese

### 11.3.1. Microstructure of Rennet-Curd Cheese

#### 11.3.1.1. The Protein Phase

Cheese is essentially a concentrated protein gel, which occludes fat, moisture, and other materials. Gelation of the milk is brought about either by :

- Hydrolysis of the casein micelle-stabilizing  $\kappa$ -casein by the action of selected acid proteinases (rennets), and the resultant slow quiescent aggregation of the destabilized micelles in the presence of calcium ions ( $\sim 3$  mM) at  $\sim 30$ – $36^\circ\text{C}$ ; (e.g., for most rennet-curd cheeses such as Cheddar, Mozzarella and Gouda)
- Acidification to the isoelectric pH of casein using lactic acid bacteria or food-grade acids/acidogens, at  $20$ – $40^\circ\text{C}$ , and resultant slow quiescent aggregation of the sensitized casein micelles e.g., for cream cheese. [A combination of acidification and rennet-hydrolysis (a smaller quantity of rennet than for rennet-curd cheeses, e.g.,  $5$ – $100$  versus  $900$ – $1000$  chymosin units per  $100$  L milk) is normally used for low-fat acid-curd cheeses such as Quark and related varieties (Schulz-Collins and Senge, 2004)]
- Non-quiescent acidification to pH  $\sim 5.4$ – $5.6$  at a high temperature ( $80$ – $90^\circ\text{C}$ ), [e.g., for Paneer, Ricotta, and some forms of Queso Blanco cheese (Farkye, 2004)].

The microstructure of milk gels and of the resultant cheeses has been studied extensively (Hall and Creamer, 1972; Kimber *et al.*, 1974; de Jong, 1978a; Brooker, 1979; Gavarić *et al.*, 1989; Kiely *et al.*, 1992, 1993; Desai and Nolting, 1995; Bryant *et al.*, 1995; Kaláb, 1995; Mistry and Anderson, 1993; Guinee *et al.*, 1998, Fenelon *et al.*, 1999; Auty *et al.*, 2001). The gelation of milk is characterized by aggregation of the rennet-altered casein micelles into interconnected clusters and forming a network in which fat globules are interspersed as loose inclusions (Gavarić *et al.*, 1989). The protein matrices of both acid-induced and rennet-induced milk gels are particulate (Gavarić *et al.*, 1989), being composed of entangled clusters of partially fused casein or *para*-casein aggregates. Continued aggregation of the casein, or *para*-casein, and expulsion of whey during cheese manufacture leads to the gradual fusion of the *para*-casein micelles and contraction of the protein gel network around the fat globules, which are, consequently, forced into closer proximity. Hence, the matrix changes from being particulate to a highly fused aggregated structure in which the fat globules and protein are microstructurally in contact (Figure 2a).

The integrity of the matrix is maintained by various intra-aggregate and inter-aggregate electrostatic and hydrophobic attractions between amino acid side groups on the caseins, and between calcium ions and organic serine phosphate groups or ionized carboxyl residues (calcium bridges) (Walstra and Van Vliet, 1986). The network is essentially continuous, extending in all directions, although some discontinuities exist at the micro-structural and macro-structural levels. Micro-structural observations made using transmission electron microscopy (TEM) suggest that the hydrolysis of *para*-casein (e.g., by rennet activity) to water-soluble peptides results in parts of the matrix losing contact with the main *para*-casein network, an occurrence that leads to discontinuities or 'breaks' in the *para*-casein matrix at the microstructural level (de Jong, 1978a). Hence, it is noteworthy that ageing of Mozzarella for 50 d results in the degradation of 50% of  $\alpha_{s1}$ -casein to  $\alpha_{s1}$ -CN (f 24–199) and an increase in the porosity of the defatted *para*-casein matrix, as observed using scanning electron microscopy (SEM) (Kiely *et al.*, 1993). Discontinuities at the macrostructural level exist in the form of curd granule junctions and, in Cheddar and related dry-salted cheese varieties, as curd chip junctions. Both kinds of junction are discernible by the naked eye in appropriately prepared sections (Brooker, 1979; Kaláb, 1979; Lowrie *et al.*, 1982; Rüegg and Blanc, 1987). Unlike the interior of the curd particles, which consists of protein and fat at a ratio corresponding closely to that for the overall cheese, the junctions are comprised mainly of casein, being almost devoid of fat. The difference in cheese composition between the interior and the surface of curd particles arises as a result of the cutting or breaking of the coagulum into curd particles, which

leads to the loss of fat globules from the freshly cut surfaces into the surrounding whey. As the protein matrix contracts and adjoining curd particles mat through their fat-depleted surface layers, these fat-depleted areas become part of the internal structure of the cheese.

Various physico-chemical changes occur in the structural components of the *para*-casein matrix during maturation; these changes are mediated by the residual rennet, microorganisms and their enzymes, and changes in mineral equilibrium between the serum and *para*-casein matrix. The type and level of the physico-chemical changes depend on the cheese variety, cheese composition and ripening conditions. These may include:

- Hydrolysis of the *para*-casein into peptides and amino acids (Upadhyay *et al.*, 2004) and of tryglycerides into fatty acids and various catabolic products such as esters, alcohols and lactones (Collins *et al.*, 2004)
- Changes in the equilibrium concentrations of calcium and inorganic phosphate between the matrix and the occluded serum, with the equilibrium being influenced by maturation time, pH and other factors such as the concentration of Na<sup>+</sup> in the moisture phase and soluble Ca (Le Graet *et al.*, 1983; Karahadian and Lindsay, 1987; Guo and Kindstedt, 1995; Guo *et al.*, 1997; Paulson *et al.*, 1998)
- Increase in hydration of the *para*-casein and physical expansion or swelling of the *para*-casein matrix, at least in Mozzarella cheese (Kindstedt, 1995; Guo and Kindstedt, 1995; Guo *et al.*, 1997; Thierry *et al.*, 1998; Boutrou *et al.*, 1999; Guinee *et al.*, 2000a, 2002)

The hydration and swelling of the casein matrix has a major influence on the structure of the fat phase and the cooking properties of the cheese, as discussed in Sections 11.3.1.2 and 11.9.

#### 11.3.1.2. Microstructure of the Fat Phase

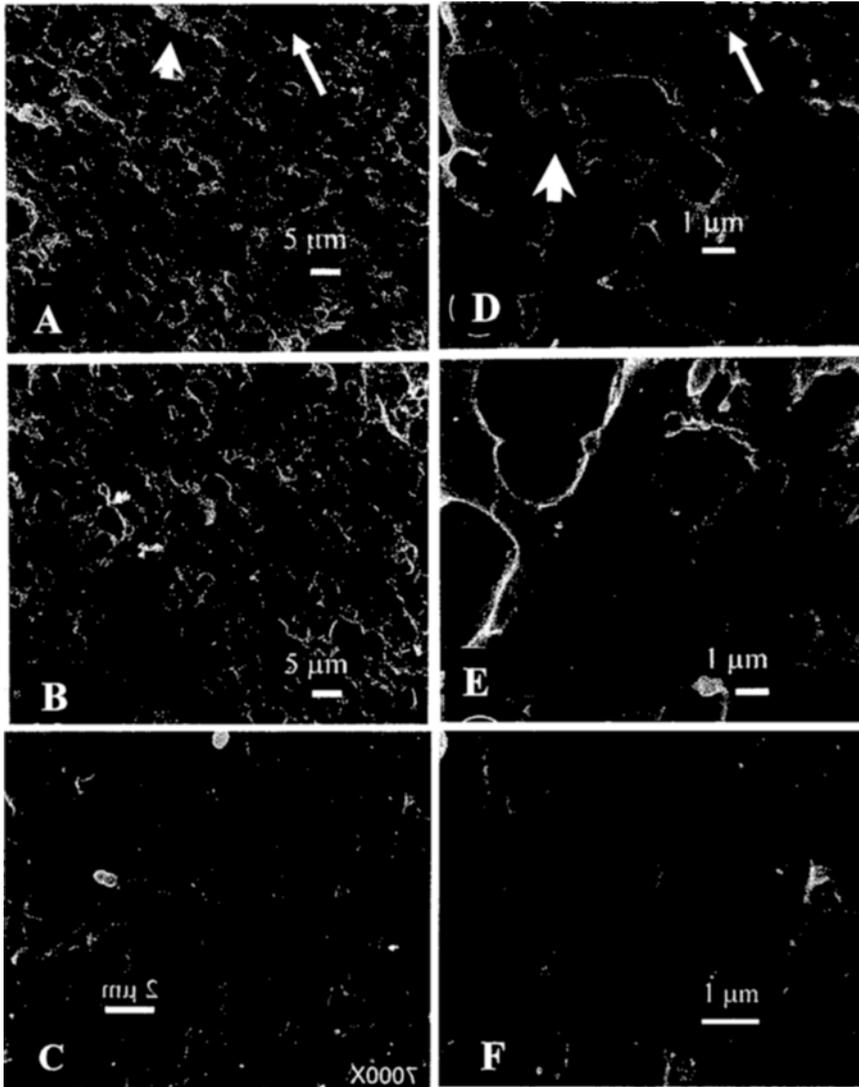
The enmeshed fat globules occupy the spaces between the protein strands and may be considered to impede physically the aggregation of the *para*-casein matrix, to a degree dependent on their volume fraction and size distribution. Consequently, a higher fat level leads to slower syneresis during manufacture (Dejmek and Walstra, 2004), and an increase in the level of MNFS in the cheese (Tunick *et al.*, 1995; Poudaval and Mistry, 1999; Fenelon and Guinee, 1999); the increase in MNFS has a major impact on cheese yield and quality, as discussed in Sections 11.4, 11.6–11.9.

Some clumping and/or coalescence of fat globules generally occur in most cheese varieties. Evidence for the clumping of fat globules in Cheddar cheese has been demonstrated clearly by both SEM and TEM

(Hall and Creamer, 1972; Kalab, 1979; Mistry and Anderson, 1993; Bryant *et al.*, 1995; Metzger and Mistry, 1995) and confocal laser scanning microscopy (CLSM) (Guinee *et al.*, 1999; Auty *et al.*, 1999). SEM micrographs of cheese samples (e.g., Cheddar) from which the fat globules had been extracted during sample preparation, reveal irregularly shaped voids in the *para*-casein matrix (Mistry and Anderson, 1993; Bryant *et al.*, 1995; Figure 11.2a). Similarly, SEM shows coalescence in Mozzarella and String cheeses in which clumped fat globules coexist with moisture as long channels between the *para*-casein fibers (Taneya *et al.*, 1992; Kiely *et al.*, 1992; McMahon *et al.*, 1993, 1999; Tunick *et al.*, 1993a; Kalab, 1995; Guinee *et al.*, 1999). TEM micrographs taken over the course of Cheddar manufacture clearly show the aggregation of fat globules, which is first notable at maximum scald and increases during cheese making as the protein network shrinks and forces the fat globules into closer contact (Kimber *et al.*, 1974; Kalab, 1995; Laloy *et al.*, 1996). In contrast to Cheddar and Mozzarella, relatively little clumping and coalescence of fat globules is evident in other cheese types such as Cheshire, Gouda (Hall and Creamer, 1972) or Meshanger cheese (de Jong, 1978a). The relatively high degree of fat globule coalescence in Cheddar and Mozzarella is probably due to the relatively large displacement of neighboring layers of protein matrix, between which fat globules and fat globule clusters are sandwiched during the cheddaring and/or kneading/stretching stages of manufacture. Such displacement can be expected to “stretch” the fat globules and, consequently, shear and disrupt their membranes.

Factors that may contribute to the clumping and coalescence of fat globules in cheese include:

- Shearing of the MFGM during cheese manufacture, as a result of stress and strains inflicted on the curd particles during cutting, stirring and curd handling operations such as dewheying, cheddaring, milling, salting, pressing and/or plasticization.
- Dehydration and contraction of the *para*-casein matrix during manufacture that force the occluded globules into closer contact (Kimber *et al.*, 1974).
- Hydration and swelling of the casein matrix during storage, at least in Mozzarella cheese, an event which is expected to shear, and rip, residual membrane from the fat globules/globule clumps (Kindstedt and Guo, 1997; McMahon and Oberg, 1999).
- A possible increase in the permeability of the MFGM during maturation due to storage-related hydrolysis of membrane components by lipoprotein lipase activity (Sugimoto *et al.*, 1983; Deeth, 1997).



**Figure 11.2.** Scanning electron micrographs of full-fat (33.0%, w/w; A, D); half-fat (17.0%, w/w; B, E) and low-fat (3.9% w/w; C, F) Cheddar cheese at low (A, B, C) or high (D, E, F) magnifications. The arrows indicate the *para*-casein matrix and the arrowheads the areas occupied by fat and free serum prior to their removal during sample preparation [modified from Guinee *et al.*, 1998 (a, b, d, e) and Fenelon *et al.*, 1999 (c, f) with permission].

- An increase in the porosity of the *para*-casein matrix, concurrently with the age-related increase in proteolysis (Tunick and Shieh, 1995), which may reduce the impedance of the matrix structure to the movement of fat.

The more extensive degree of coalescence of fat in Mozzarella and String cheeses compared to Cheddar reflects the shearing of the MFGM during plasticization and elongation of the curd. Plasticization that involves kneading and heating of curd to  $\sim 57^{\circ}\text{C}$  in hot water, is conducive to deformation and disruption of the MFGM and aggregation of globular and non-globular fat. Similarly, elongation of plasticized curd in the manufacture of String cheese results in the deformation (and probably coalescence) of the fat globules lying between contiguous layers of the *para*-casein matrix, to a degree that increases with elongation (Taneya *et al.*, 1992).

At the temperatures used in the manufacture of cheese ( $\sim 30\text{--}55^{\circ}\text{C}$ ) much, or all, of milk fat is liquid (Wright *et al.*, 2002) and can therefore flow and aggregate, leading to coalescence on the application of stress. A significant portion of the fat ( $\sim 20\text{--}30\%$  total) may be liquid at the ripening temperatures used for Cheddar or Mozzarella ( $\sim 4\text{--}7^{\circ}\text{C}$ ) (Wright *et al.*, 2002), and will aggregate, leading to coalescence. Thus, increasing the liquid-to-solid fat ratio, by increasing the ratio of low melting point fat fraction (olein) to the high melting point (stearin), results in a higher level of free oil in Mozzarella cheese made from recombined milk prepared by homogenizing skim milk and fat fractions (Rowney *et al.* 2003).

In addition to protein and fat, the matrix occludes moisture and its dissolved solutes and enzymes, and bacteria (Kimber *et al.*, 1974; Laloy *et al.*, 1996). The starter and non-starter bacteria appear to attach, *via* filaments from their cell walls, to the casein matrix (Kimber *et al.*, 1974) and are concentrated near the fat-casein interface (Laloy *et al.*, 1996; Haque *et al.*, 1997). The concentration of bacteria at the fat-casein interface may have several potential consequences (Laloy *et al.*, 1996):

- Concentration of intracellular bacterial peptidases in the vicinity of the protein-fat interface following bacterial lysis
- A more heterogeneous distribution of starter bacteria in cheese at the microstructural scale, as the level of fat is reduced owing to the concomitant reduction in fat/casein surface area, and
- A more uneven distribution of starter cell proteinase/peptidase activity in reduced-fat cheese and possible restricted access of substrates (polypeptides/peptides released by the action of coagulant, etc.) to enzymes

Hence, the location of bacteria in cheese at the fat-casein interface may be important in relation to the growth dynamics of starter and non-starter bacteria in cheese and their effects on cheese maturation (*cf.* Sections 11.5, 11.7–11.8).

### 11.3.2. Microstructure of Pasteurized Processed Cheese Products (PCPs) and Analogue Cheese Products (ACPs)

Microstructural studies on PCPs or ACPs indicate that the structure is that of a concentrated emulsion of discrete, rounded fat droplets of varying size in a hydrated protein matrix (Kimura *et al.*, 1978; Taneya *et al.*, 1980; Rayan *et al.*, 1980; Heertje *et al.*, 1981; Lee *et al.*, 1981; Kalab *et al.*, 1987; Tamime *et al.*, 1990; Savello *et al.*, 1989; Kalab, 1995; Guinee *et al.*, 1999). The fat and *para*-casein are distributed more homogeneously and the matrix is finer, being less compact and fused, than in natural rennet-curd cheese. High resolution TEM (e.g., 60 000 ×) shows that the protein phase consists of varying proportions of individual *para*-caseinate particles and strands, which are probably formed through association of *para*-caseinate particles. The individual particles (20–30 nm in diameter) may correspond to casein sub-micelles released from the *para*-casein micelles in the matrix of the natural cheese as a result of sequestration of calcium by the emulsifying salt (ES). The rheology and texture characteristics of the cheese vary with the proportions of strands to individual particles, with hard PCPs containing a high level of long protein strands (e.g., ~100 versus 25 μm), which form a matrix and soft PCPs usually consisting mainly of individual particles.

Compared to natural cheese, there is less clumping or coalescence of fat globules in PCPs and ACPs. Consequently, the mean fat globule size tends to be generally smaller (Sutheerawattananonda and Bastian, 1995), although it varies depending on the type and level of ES, types and levels of milk protein added, processing time and extent of shear (Rayan *et al.*, 1980; Kaláb *et al.*, 1987, 1991a; Savello *et al.*, 1989; Tamime *et al.*, 1990). Generally, for most emulsifying salts, the fat globule size decreases as the processing time at a high temperature increases, [e.g., up to 40 min (Kaláb *et al.*, 1987; Ryan *et al.*, 1980)]. The *para*-caseinate membrane of the emulsified fat globules appears to attach to the matrix strands, thereby contributing to the continuity of the matrix. The incorporation of emulsified *para*-caseinate-coated fat globules, which can be considered as pseudo-protein particles, into the new structural matrix may be considered as increasing the effective protein concentration (van Vliet and Dentener-Kikkert, 1982; Marchesseau *et al.*, 1997; Michalski *et al.*, 2002).

The degree of fat emulsification and the fat globule size have a major impact on the rheology and cooking properties of PCPs and ACPs (Guinee *et al.*, 2004a)

### 11.3.3. Effect of Fat Level on Microstructure

The effects of fat content on the microstructure of Cheddar (Mistry and Anderson, 1993; Bullens *et al.*, 1994; Baer *et al.*, 1995; Bryant *et al.*, 1995; Desai and Nolting, 1995; Metzger and Mistry, 1995; Drake *et al.*, 1996a,b; Guinee *et al.*, 1998, 2000a) and Mozzarella (McMahon *et al.*, 1996, 1999) have been evaluated in a number of studies but little information is available on effect of fat content on the structure of other rennet-curd cheese varieties.

Increasing the fat content of Cheddar results in a reduction in the volume fraction and continuity of the casein matrix, which becomes more interrupted by fat globules (Figure 11.2). Concomitantly, the fat globules become more numerous and varied in size and shape, and the degree of clumping and coalescence of the fat globules increase. The increased degree of fat globule aggregation is expected because the number of encounters of the fat globules within a given volume of the casein matrix increases as the enveloping protein matrix contracts during manufacture and as the curd particles undergo deformation during the various stages of cheese making. Conversely, as the fat content of cheese is reduced there are longer stretches of uninterrupted casein matrix and the fat globules become more uniformly dispersed and less clumping is evident. Some fat mimetics (e.g., Novagel<sup>™</sup>, Dairy Lo<sup>™</sup>, ALACO PALS<sup>™</sup>) have been found to enhance the uniformity of the fat distribution in reduced-fat Cheddar cheese (Drake *et al.*, 1996a).

Similarly, a comparison of regular (Oberg *et al.*, 1993; McMahon *et al.*, 1999) and low-fat Mozzarella (LFMC) (McMahon *et al.*, 1996) showed that reducing the fat content gives a denser casein matrix and a lower degree of fat coalescence. Indeed, in low-fat Mozzarella (e.g., < 4%, w/w), there are insufficient fat globules to keep the casein strands apart as the curd is forming (McMahon *et al.*, 1993). Owing to the high degree of casein aggregation, the matrix is extremely compact and the number and width of the serum-fat channels between the protein layers is reduced markedly compared to the regular part-skim Mozzarella curd (~17%, w/w, fat). Consequently, the matrix of low-fat Mozzarella has a low moisture-retaining capability and the cheese has a relatively low level of MNFS. The change in microstructure that accompanies fat reduction adversely affects the heat-induced functionality of Mozzarella (Section 11.9). McMahon *et al.* (1996) reported that the addition of Novagel<sup>™</sup> (a blend of microcrystalline cellulose and guar gum with a particle size of 10 to 100  $\mu\text{m}$ ) to the cheese milk resulted

in the formation of large amorphous particles in low-fat Mozzarella. These large particles had the effect of impeding the degree of *para*-casein aggregation during curd manufacture and plasticization. Consequently, the Novagel<sup>™</sup> particles resulted in the formation of relatively large serum channels (e.g., up to 300  $\mu\text{m}$ ) between the protein fibers and a higher moisture content, compared to the control low-fat Mozzarella.

#### 11.3.4. Effect of Fat Emulsification on Microstructure

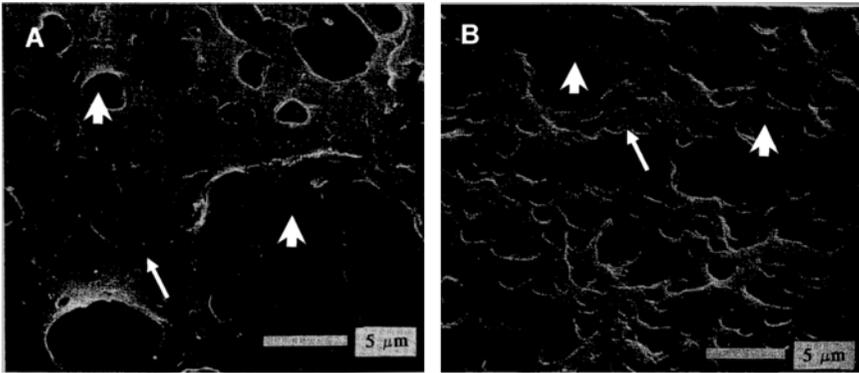
The degree of fat emulsification in natural rennet-curd cheeses may be increased by homogenization or microfluidization of the cheese milk, or of the cream used to standardize the cheese milk. Microfluidization is also a particle size reduction process that is used mainly in the manufacture of products such as antibiotic dispersions, parenteral emulsions, and diagnostics. It is generally accepted that for the application of equivalent pressures to the milk, microfluidization gives a lower mean fat globule diameter (e.g., 0.03–0.3  $\mu\text{m}$  compared to 0.5–1.0  $\mu\text{m}$ ) and a narrower size distribution than homogenization (typically 1–2  $\mu\text{m}$ ). Moreover, the fat globule membrane in microfluidized milk has a higher proportion of fragmented casein micelles than homogenized milk and has little, or no, whey protein. Additionally, the degree of fat emulsification in acid-curd natural cheeses may be increased by shearing of the curd after whey separation (Mahaut and Korolczuk, 2002).

Homogenization of milk is an integral part of the manufacturing process for soft, high-fat, acid-curd cheeses such as Cream cheese, as it prevents creaming (flocculation of fat globules) during the relatively long gelation time (e.g., >4 h) and contributes to the formation of a homogeneous, viscous, creamy texture in the end product (Mahaut and Korolczuk, 2002). In contrast, milk or cream is not normally homogenized in the manufacture of hard and semi-hard rennet-curd cheeses, as it leads to curds that do not knit/mat well together and to crumbly cheese. The defects are probably associated with the formation of casein-covered emulsified fat particles, which are more stable than the native fat globules to coalescence during manufacture. The absence of free fat that acts as a lubricant on protein surfaces (Marshall, 1990), probably impairs the ability of layers of the casein matrix on the surface of neighboring curd particles to deform and fuse. Moreover, the casein-coated emulsified fat particles, which may be considered to behave as pseudo-protein particles (van Vliet and Dentener-Kikkert, 1982), probably lead to more interactions between the adjoining layers of the casein matrix. This in turn probably impedes the ability of the casein matrix to deform and curd particles to flow together after whey removal and during cheddaring, molding, and/or pressing. In addition to the above, homogenization of milk or cream leads to increased moisture

retention by the casein matrix (Guerzoni *et al.*, 1999) and higher moisture cheeses (Jana and Upadhyay, 1992; Oommen *et al.*, 2000; Nair *et al.*, 2000). Homogenization of cheese milk also increases the sensitivity of the fat to lipolysis (Geurts *et al.*, 2003), which alters the flavor balance and for many cheeses would be undesirable.

Homogenization is essential in the manufacture of rennet-curd cheeses from recombined milk (homogenized blend of anhydrous butter oil and reconstituted skim milk powder) and the above defects are minimized *inter alia*, by the use of a very low pressure (e.g., 3–5 MPa) (Jana and Thakar, 1996). Sometimes, homogenization of milk may be used advantageously, for example, to give a whiter color in the resultant cheeses (e.g., Danablu) (Cantor *et al.*, 2004), or to increase the accessibility of the fat to fungal lipases and thereby increase the formation of fatty acids and their derivatives (e.g., methyl ketones) in cheeses where lipolysis is important for flavor development, (e.g., Blue cheese) (Collins *et al.*, 2004). Much work has been undertaken to evaluate the potential of homogenization of milk and/or cream as a technique for improving the texture and heat-induced functionality of reduced-fat Cheddar and Mozzarella cheeses (Oommen *et al.*, 2000). The basis for such potential is that homogenization increases the number of fat particles, which may be considered as spacers between, and to limit interaction between, neighboring layers of the casein matrix. However, this effect may be mitigated by the ability of the casein-covered fat globules to interact with, and thereby strengthen, the casein matrix of the cheese (van Vliet and Dentener-Kikkert, 1982). Moreover, the effectiveness of emulsified fat globules as spacers is undoubtedly dependent on their size distribution and spatial distribution within the casein matrix, and it is conceivable that below a critical mean size they have little, or no, effect. Consequently, there is considerable discrepancy between published studies *vis-à-vis* the results of homogenization on cheese texture and functionality, depending, *inter alia*, on the homogenization conditions, including pressures, number of stages, and temperature used (see Jana and Upadhyay, 1991, 1992, 1993; Jana and Thakar, 1996; Sections 11.8 and 11.9).

Homogenization of cheese milk, at a pressure in the range 2.6–30 MPa, causes a more uniform dispersion of fat globules, and a marked reduction in both the size and the degree of clumping and coalescence of fat globules in Cheddar and Mozzarella (Figure 11.3; Metzger and Mistry, 1995; Baer *et al.*, 1995; Tunick *et al.*, 1997; Rudan *et al.*, 1998b; Guinee *et al.*, 2000c; Rowney *et al.*, 2003); a similar effect is generally observed on homogenization of cream at 21 MPa prior to addition skim milk (Metzger and Mistry, 1995; Poudaval and Mistry, 1999; Oommen *et al.*, 2000), although the effect varies with homogenization pressure (Nair *et al.*, 2000). Moreover, the *para*-casein matrix of full-fat Cheddar made from homogenized milk is more continuous and



**Figure 11.3.** Scanning electron micrographs of 26 week-old reduced-fat (~18.0% w/w) Cheddar cheese prepared from non-homogenized milk (A) or from milk consisting of skim milk and cream homogenized at first- and second-stage pressures of 17.3 and 3.4 MPa (B), respectively. The arrows show the *para*-casein matrix and the arrowheads the fat globules. The micrographs show the presence of elongated fat globules of variable size unevenly dispersed in A, and relatively small fat globules evenly dispersed in the *para*-casein matrix in B (reproduced from Metzger and Mistry, 1995, with permission).

occupies a greater volume than, that of the corresponding control, which contains large protein-deficient areas occupied by pools of coalesced fat (Figure 3).

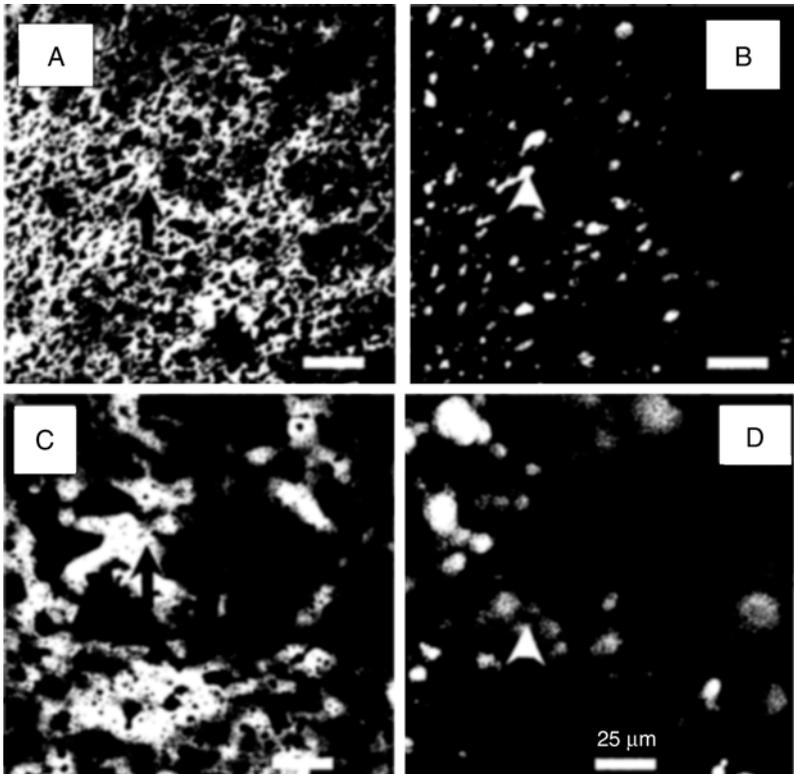
The manufacture of PCPs and ACPs involves the application of high temperature, high shear and the use calcium-chelating salts. These conditions assist in the dispersion of free fat and the conversion of the insoluble calcium *para*-casein to a more hydrated sodium *para*-caseinate, which coats the surface of dispersed free fat droplets and emulsifies them (Guinee *et al.*, 2004a). Consequently, the fat droplets in processed cheese are discrete with little evidence of clumping or coalescence (Guinee *et al.*, 1999). The actual size depends on the formulation and processing conditions, (e.g., addition of emulsifying salts and milk proteins, processing time, and extent of shear) (Rayan *et al.*, 1980; Carić *et al.*, 1985; Savello *et al.*, 1989).

### 11.3.5. Effect of Fat on Heat-induced Changes in Microstructure

On cooking, cheese is heated to a high temperature (e.g., 80–100°C), which alters its microstructure to a level dependent on the level of fat, degree of fat emulsification, nature of the fat globule membrane, and, hence, cheese type. In some cheese varieties, including full-fat Cheddar and high-fat (28% w/w) and stirred-curd Mozzarella, heating has been found to cause extensive clumping and coalescence of fat globules/pools (Paquet and Kalab, 1988;

Auty *et al.*, 1999; Guinee *et al.*, 2000c) and a concomitant increase in the heterogeneity of the distributions of the fat and *para*-casein phases (Figure 11.4). The *para*-casein matrix of Mozzarella tends to loose its orientation and become more compact on heating; this effect is attributed to the depletion of fat between adjoining layers of the protein matrix owing to fat liquefaction and coalescence, and its seepage from the cheese mass.

Changes in the distribution of fat on baking half-fat Cheddar and reduced-fat (15%, w/w) Mozzarella are similar to those noted for their full-fat counterparts. However, the degree of aggregation of fat globules and the size of the coalesced fat particles in the reduced-fat cheeses are generally smaller than in full-fat cheeses. This trend undoubtedly reflects the lower



**Figure 11.4.** Confocal laser scanning micrographs of 5 day-old full-fat Cheddar cheese before heating (A, B) and after heating to 95°C and then cooling to room temperature (C, D). The micrographs show protein (black arrows in A, C) and fat (white arrow heads in B, D) as light areas against a dark background. Bar = 25 µm (modified from Guinee *et al.*, 2000b).

volume fraction of the fat phase in reduced-fat cheese (van Boekel and Walstra, 1995). The heat-induced coalescence of fat in cheese suggests a tendency towards phase separation and is consistent with the increase in the leakage of fat or oiling-off that occurs on baking or grilling cheese.

In contrast to natural cheeses prepared from unhomogenized milk, heating generally has little influence on the microstructure (distribution of fat and protein) of pasteurized PCPs (Paquet and Kalab, 1988) or on that of full-fat Cheddar cheese prepared from homogenized milk (Guinee *et al.*, 2000c). The relatively high thermo-stability of these products against fat coalescence is probably due to the higher degree of emulsification prior to heating and a higher heat stability of the artificial fat globule membrane compared to the native fat globule membrane.

### 11.4. Effect of Fat on Cheese Yield

Milk fat contributes directly and indirectly to cheese yield (Table 11.3). The direct contribution of fat to cheese yield is clearly reflected by prediction equations, which relate cheese yield to the concentrations, and recoveries, of milk fat and protein (Fox *et al.*, 2000; Melilli *et al.*, 2002). An example of such an equation is the modified van Slyke formula, (Fenelon and Guinee, 1999):

$$Y_p = \frac{\left[ F \left( \frac{\% FRC}{100} \right) + (CN - a) + \left( WP_{um} \times \frac{\% WPD_{pm}}{100} \right) \right] \times (1 + SNFP)}{1 - \left( \frac{\text{reference moisture content}}{100} \right)}$$

where, *F* and *CN* are the percentages of fat and casein in the cheese milk (with added starter culture), % *FRC* = percentage fat recovery, *a* =

**Table 11.3.** Effect of Fat Content of Milk on the Yield of Cheddar Cheese and Fat Recovered in Cheese<sup>a</sup>

Fat in milk (%, w/w)	Actual yield (kg/100 kg milk)	Predicted yield <sup>b</sup> (kg/100 kg milk)	Dry matter yield (kg/100 kg milk)	Fat recovered to cheese (% of total)
0.54	6.37	6.47	3.43	80.84
1.5	7.49	7.58	4.29	87.16
2.00	8.09	8.21	4.79	89.48
3.33	9.50	9.61	5.92	87.84

<sup>a</sup> Compiled from data of Fenelon and Guinee (1999)

<sup>b</sup> Predicted using modified Van Slyke formula, as described in text.

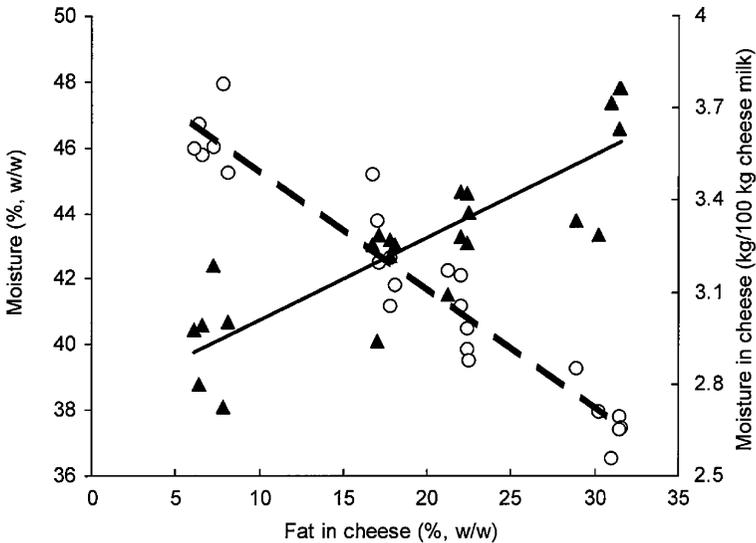
coefficient for casein loss (typically 4% of total casein);  $WP_{um}$  = percentage whey protein in the unpasteurized milk;  $\%WPD_{pm}$  = percentage of total whey protein denatured on pasteurization; and  $SNFP$  = cheese solids non-fat-non-protein (e.g., lactates, ash) as a percentage of cheese dry matter. Actual yield and predicted yield (as determined by the above equation) of Cheddar cheese from milk with fat ranging from ~0.5 to 3.4% (w/w), were closely correlated, with yield increasing at a rate 1.16 kg per 100 kg milk for every 1% increase in the fat content of the milk.

Recovery of fat in Cheddar cheese increases significantly on raising the fat content in the cheese milk from 0.5 to 2.7%, w/w, and thereafter decreases as the fat is increased further to 3.3%, w/w (Table 11.3) (Fenelon and Guinee, 1999). A similar trend was noted by Banks and Tamime (1987) who reported that the recovery of fat in Cheddar cheese manufacture increased to a maximum as the casein-to-fat ratio (CFR) was raised from 0.65 to 0.72, and decreased as the CFR was raised further to 0.75. The increase in fat recovery with milk fat content to 2.7%, w/w, may be due to the associated increase in the extent of clumping and coalescence of fat globules in the cheese milk during gelation and in the curd during cheese making (Section 11.4). The probable consequence of this partial clumping is an increase in the effective size of the fat globules (clumps), which, in effect, impedes their flow and escape through the pores of the surrounding *para*-casein matrix into the whey. A tentative explanation for the reduction in fat recovery at higher fat levels (>2.7%, w/w) is excessive clumping, which leads to coalescence and the formation of free fat that easily permeates the *para*-casein matrix and is lost into the whey.

The level of fat recovered during cheese manufacture is also influenced by cheese variety, which determines the type and intensity of processes to which the curd is subjected, which in turn influence the level of damage to the MFGM. Hence, the percentage for fat recovery reported for Mozzarella is markedly lower than that for Cheddar, (e.g., 80% *versus* 88% in pilot-scale studies) (Fenelon *et al.*, 1999; Guinee *et al.*, 2000b). The lower fat recovery for LMMC compared to Cheddar may be attributed to the high loss of fat during the kneading and stretching of the curd in hot water (plasticization). The high loss of fat during the plasticization process is consistent with the increase in the degree of coalescence that accompanies shearing of the curd, as observed by CLSM (see Fox *et al.*, 2000). In contrast, homogenization or microfluidization of cheese milk or cream stabilizes the fat to coalescence during manufacture and thereby reduces the level of fat lost in the whey and in the stretch water (Quarne *et al.*, 1968; Lelievre *et al.*, 1990; Lemay *et al.*, 1994; Lemay *et al.*, 1994; Metzger and Mistry, 1994; Oommen *et al.*, 2000) during the manufacture of plasticized cheeses. The increases in the recovery of fat and moisture (as discussed

in Section 11.2) associated with the homogenization of milk or cream lead to an increased yield of cheese, to a degree dependent on milk composition, cheese type, and homogenization conditions (number of stages, pressure, and temperature) (Jana and Upadhyay, 1991, 1992); (e.g., the percentage increase in yield over the control for Cheddar  $\sim 32\%$ , w/w, fat) from unhomogenized milk was  $\sim 4$ , 6 and 8% when cream used for milk standardization was homogenized at a total pressure of  $\sim 7$ , 10 or 14 MPa (Oommen *et al.*, 2000). How homogenization of milk increases the retention of moisture in cheese is unclear; however, a tentative explanation may be the increase in the firmness of the rennet-induced gels (at a given time after rennet addition) on homogenization of the milk (Guinee *et al.*, 1997; Nair *et al.*, 2000). The moisture content of cheese increases markedly as the firmness of the gel at cutting is increased (Mayes and Sutherland, 1989; Guinee *et al.*, 2004b). Thus, as the firmness of the gel at cutting was not standardized in most cheese making studies, the expected increase in curd firmness at cutting may contribute, at least partly, to the higher level of moisture in cheese made from homogenized milks. However, in contrast to the above, other investigators have reported that homogenization impairs curd-forming properties, leading to a weaker gel and a longer set-to-cut time (Jana and Upadhyay, 1992, 1993). Other factors that contribute to increased moisture retention in curds from homogenized milk may include associated alterations in the microstructure of the curd (Tunick *et al.*, 2002) and permeability of the matrix to moisture.

Fat also contributes indirectly to cheese yield, as its presence in the *para*-casein curd matrix affects the degree of matrix contraction and hence moisture content and cheese yield. The occluded fat globules physically limit contraction of the surrounding *para*-casein network and therefore reduce the extent of syneresis. Thus, it becomes more difficult to expel moisture as the fat content of the curd is increased. Consequently, the moisture-to-casein ratio generally increases unless the cheese making process is modified to enhance casein aggregation, (e.g., by increasing the scald temperature) (Gilles and Lawrence, 1985; Fenelon and Guinee, 1999). Owing to its negative effect on syneresis, fat indirectly contributes more than its own weight to actual cheese yield, (e.g., Cheddar cheese yield increases by  $\sim 1.16$  kg/kg milk fat). The greater than *pro rata* increase is due to the concomitant increase in the level of moisture associated with the cheese protein as reflected by the positive relationship between milk fat level and MNFS (Figure 11.1). Hence, while the percentage moisture in Cheddar cheese is inversely related to its fat content, the weight of cheese moisture from a given weight of cheese milk increases as the fat content of the cheese is increased (Figure 11.5). Moreover, the increase in the moisture-to-protein ratio with fat content contributes indirectly to cheese



**Figure 11.5.** Effect of fat content on the percentage moisture in Cheddar cheese (○) and the weight of Cheddar cheese moisture obtained from 100 kg cheesemilk (▲) (drawn from data of Fenelon and Guinee, 1999; Guinee *et al.*, 2000a).

yield due to the presence of dissolved solids, including native whey proteins,  $\kappa$ -casein glycomacropeptide, lactate, and soluble salts. However, if the level of MNFS is maintained constant (e.g., by process modifications), fat contributes less than its own weight to cheese yield (i.e.,  $\sim 0.9$  kg/kg for Cheddar), due to the fact that  $\sim 10\%$  of the fat in milk is normally lost in the whey during Cheddar manufacture.

The dry matter cheese yield ( $Y_{dm}$ ) increases with the level of milk fat but at a lower level than actual yield ( $Y_a$ ), [i.e.,  $\sim 0.93$  vs.  $1.16$ , kg/kg milk fat for Cheddar (Table 11.3; Fenelon and Guinee, 1999)]. The difference between the increase in  $Y_a$  and  $Y_{dm}$  per unit weight of fat in milk (i.e.,  $0.23$  kg/kg milk fat for Cheddar) is due to the fact that  $Y_{dm}$  excludes the effect of milk fat on cheese moisture (i.e.,  $0.24$  kg/kg fat for Cheddar) whereas  $Y_a$  incorporates it. However, the increase in  $Y_{dm}$  per kg of milk fat is greater than expected based on the corresponding increase in the weight of cheese fat per kg of milk fat (i.e.,  $0.90$  kg/kg; Table 11.3). The difference (i.e.,  $0.03$  kg/kg milk fat) in the extent of the increase between  $Y_{dm}$  and weight of fat in cheese per unit weight of fat in the milk, may be attributed to the increased weight of the soluble portion of the SNFP (which forms a major part of the dissolved solids) in the cheese as the fat content increases (Table 11.3). The

latter trend in turn is due to the increase in cheese moisture per kg cheese milk as the level of fat in milk increases (Figure 11.5). However, the direct contribution of fat to  $Y_{dm}$  is less than its own weight in milk due to the loss of fat in cheese whey ( $\sim 10\%$  total).

### 11.5. Effect of Fat on Cheese Microbiology

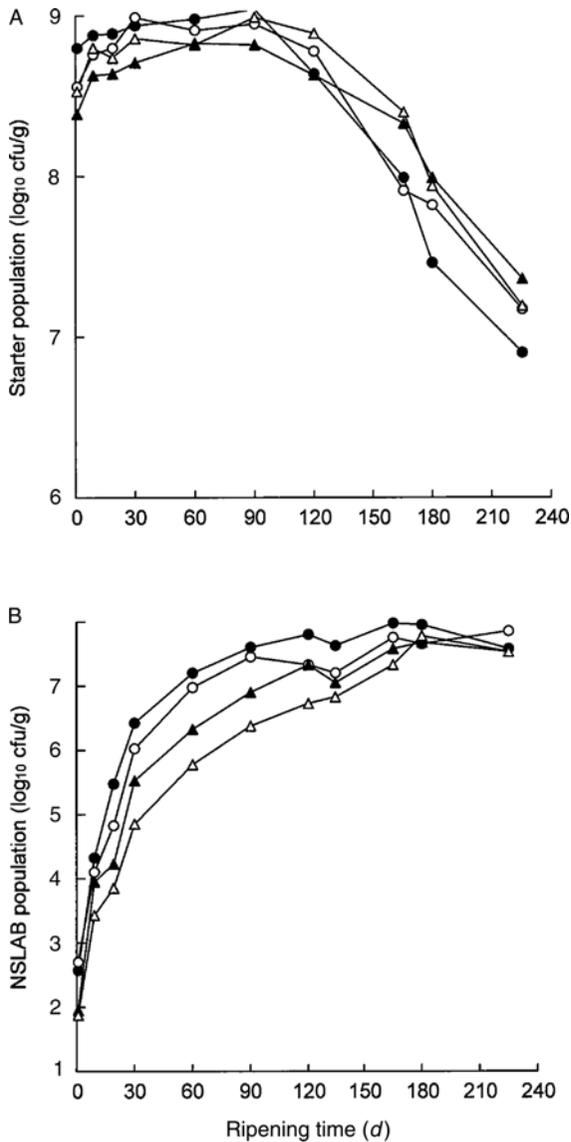
During cheese ripening, the population of starter bacteria generally decreases while the number of non-starter lactic acid bacteria (NSLAB) generally increases; these changes are well documented for many full-fat rennet-curd cheese varieties, (e.g., Cheddar) (Cromie *et al.*, 1987; Jordan and Cogan, 1993; McSweeney *et al.*, 1993; Lane *et al.*, 1997; Haque *et al.*, 1997; Beresford and Williams, 2004).

However, comparatively little information is available on the effect of fat content on the dynamics of starter and NSLAB populations in cheese. Laloy *et al.* (1996) reported that the number of starter cells in full-fat curd prior to pressing was 4-fold to 10-fold higher than the corresponding population in fat-free Cheddar curd, depending on the starter strain used. The authors suggested that the higher number of starter cells in the full-fat cheese might be attributable to:

- The association between starter lactococci and fat globules,  $\sim 90\%$  of which are retained in the curd
- The physical impedance to syneresis by the fat globules, which, in effect, act as ‘stoppers’ in the pores of the *para*-casein matrix and thereby reduce the loss of starter cells in the whey exuding from the curd; the retained starter cells aggregate around the fat globules

In agreement with the trend noted by Laloy *et al.* (1996), Fenelon *et al.* (2000a) reported that the starter cell count in full-fat Cheddar (FFC; 32.5%, w/w) at 1 d was significantly higher than that in low-fat Cheddar (LFC, 6.3%, w/w). However, the starter population in the FFC declined more rapidly and was significantly lower than that in the LFC at 180 d (Figure 11.6a). In contrast, Haque *et al.* (1997) reported similar populations ( $\sim 3.2 \times 10^8$  cfu/g) of starter lactococci in LFC and FFC at 1 d but counts in the LFC decreased more rapidly during maturation; the populations in the LFC and FFC at 180 d were  $\sim 6.3 \times 10^3$  and  $2.5 \times 10^4$  cfu/g, respectively.

The number of NSLAB in Cheddar decreases with fat content (Figure 11.6b), with the count in LFC (5%, w/w) being significantly lower than that in FFC (33%, w/w) (Haque *et al.*, 1997; Fenelon *et al.*, 2000a). The decrease in NSLAB as the fat content of cheese is reduced may be due to a number of factors including:



**Figure 11.6.** Age-related changes in the populations of starter (A) and non-starter bacteria (B) in full-fat (30.4%, w/w; ●); reduced-fat (21.9%, w/w; ○); half-fat (17.2%, w/w; ▲); and low-fat (7.2%, w/w; △) Cheddar cheese. The values presented are the means of three replicate trials (drawn from data of Fenelon *et al.*, 2000a).

- The reduction in the concentration of milk fat globule membrane-associated glycoproteins. Mesophilic lactobacilli possess glycoside hydrolases and may be able to release sugars from the glycomacro-peptide of casein and glycoproteins of the fat globule membrane (Beresford and Williams, 2004). The released sugars may be a source of energy for propagation
- The lower level of MNFS (Lane *et al.*, 1997)

## 11.6. Effect of Fat on Proteolysis

Proteolysis in cheese has been studied extensively and reviewed (Fox and Wallace 1997; McSweeney, 2004; Upadhyay *et al.*, 2004). It contributes directly to flavor, *via* the formation of peptides and free amino acids (FAA), and indirectly *via* the catabolism of free amino acids to various compounds including amines, acids, thiols (Curtin and McSweeney, 2004). Proteolysis directly affects the level of intact casein, which is a major determinant of the fracture and functional properties, and of cheese texture (de Jong, 1978b; Creamer and Olson, 1982; Creamer *et al.*, 1982; Guinee, 2003; Brown *et al.*, 2003).

Most studies on proteolysis have focused on full-fat cheese or half-/reduced-fat cheeses, with little systematic comparison on the effects of incremental fat reduction, especially in cheese varieties other than Cheddar.

### 11.6.1. Primary Proteolysis

Those studies in which the effect of fat on primary proteolysis has been studied indicate that the effect depends on the level of MNFS in the cheese.

#### 11.6.1.1. Cheese with Similar Levels of MNFS

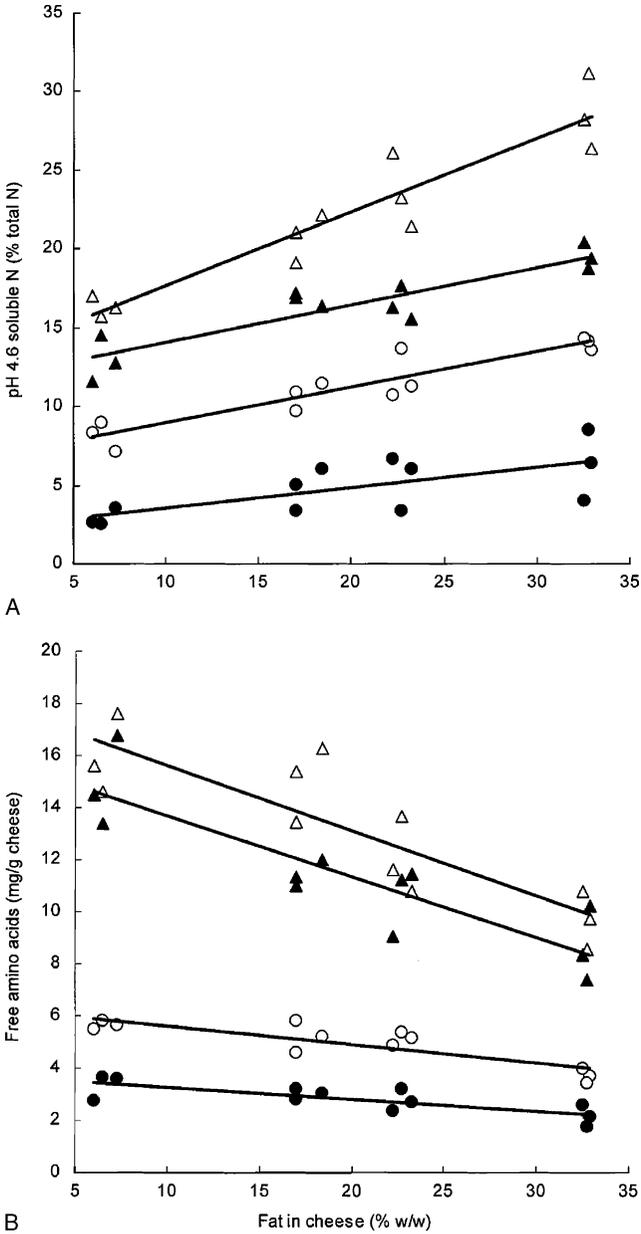
Rank (1985) investigated the effects of fat level, in the range 13.5–30.6%, w/w, on proteolysis in Colby cheese for which alterations were made to the manufacturing protocol of the low-fat cheese so as to give a level of MNFS similar to that in the full-fat cheese. After 6 to 8 months storage at 4°C, the concentration of  $\alpha_{S1}$ -casein in the lowfat cheese (13.5% w/w fat) was only slightly lower than that in the full-fat cheese (30.6%, w/w, fat). Similarly, Michaelidou *et al.* (2003a, b) reported that a large reduction in the fat content of Feta-cheeses (from ~29 to 7%, w/w) or Kefalograviera-cheeses (from ~31 to 10%, w/w) had little effect the type or level of peptides detected by urea-PAGE.

Banks *et al.* (1989) found that a 50% reduction in the fat content of Cheddar cheese only slightly reduced the level of water-soluble N at 2 (9.9 vs. 11.8 g/100g N) or 4 (15.3 vs. 17.8 g/100g N) months. Likewise,

only relatively small decreases (0 to 3 g /100 g N) in the level of pH 4.6-soluble or water-soluble N were reported for Herrgård's and Drabant cheeses (Ardö, 1993), Feta-type cheese (Michaelidou *et al.*, 2003a), or Kefalograviera-type cheese (Michaelidou *et al.*, 2003b) for large reductions in fat content (40–70%) when the level of MNFS in the reduced-fat and full-fat cheeses were similar. Moreover, the use of an adjunct culture in the manufacture of the reduced-fat cheeses eliminated the difference in the level of WSN between the full-fat and reduced-fat Kefalograviera-type and Feta-type cheeses (Michaelidou *et al.*, 2003a, b). Small differences in proteolysis in cheeses of different fat content, despite the large effect of fat on gross composition (i.e., fat, protein, and moisture), indicate the importance of MNFS as a major compositional factor controlling proteolysis in, and quality of, cheese (Creamer, 1971; Thomas and Pearce, 1981; Lawrence *et al.*, 2004). The relatively minor effect of fat on proteolysis may be attributed in part to alterations to the cheese making procedures, which minimized the difference in the level of MNFS between full-fat and reduced-fat cheeses. Hence, studies on retail Cheddar cheeses have shown no significant relationship between the levels of fat and MNFS or between fat content and the levels of primary or secondary proteolysis (Banks *et al.*, 1992; Fenelon *et al.*, 2000b).

#### 11.6.1.2. Cheeses with a Different Level of MNFS

In contrast to the above, other studies (Fenelon and Guinee, 2000; Fenelon *et al.*, 2000a) showed that the level of fat, in the range 6–33%, w/w, had a marked influence on the level of proteolysis in Cheddar cheese manufactured using identical conditions but with a different level of MNFS (Table 11.1). The mean level of primary proteolysis throughout the 225 d ripening period, as measured by the percentage of total N soluble at pH 4.6 (pH 4.6 SN %TN), decreased significantly (from ~30 g/100 g total N for cheese with ~33%, w/w, fat to 15 g/100 g total N for cheese with ~6%, w/w, fat at 225 d) as the fat level was reduced (Figure 11.7a). The decrease in proteolysis was expected owing to the parallel decrease in MNFS (~56–49%, w/w; Figure 11.1) and ratio of residual rennet activity to protein (Fenelon and Guinee, 2000; Fenelon *et al.*, 2000a). Moreover, the higher volume fraction of the protein in the reduced-fat cheese may be conducive to a higher degree of protein interaction, which in turn could restrict the availability of the casein to chymosin and other proteinases. However, the level of pH4.6-soluble N per 100 g cheese (pH4.6 SN %TN) was not significantly influenced by fat content. This suggests that the reduction in pH4.6SN%TN as the fat content decreased was compensated for by the concomitant increase in the protein content of the reduced-fat cheese. Michaelidou *et al.* (2003a, b) reported similar trends for Feta-type and Kefalograviera-type cheeses.



**Figure 11.7.** Effect of fat content on the levels of pH 4.6-soluble N (A) and free amino acids (B) in Cheddar cheeses aged for 30 (●), 90 (○), 180 (▲), or 225 (△) days (drawn from data of Fenelon and Guinee, 1999; Guinee *et al.*, 2000a).

Variation in the level of fat also leads to differences in the level of primary proteolysis as monitored by PAGE. Reducing the fat content resulted in higher levels of intact  $\alpha_{s1}$ -casein and  $\beta$ -casein in Cheddar (Fenelon and Guinee, 2000) and Mozzarella cheese (Tunick *et al.*, 1993b, 1995). This was expected because of the inverse relationship between the levels of fat and protein in cheese. However, for a given protein content, reduction in the level of fat led to more extensive degradation of  $\beta$ -casein and the accumulation of  $\gamma$ -caseins (Fenelon and Guinee, 2000). The increase in the concentration of  $\gamma$ -caseins was attributed to the higher pH in the reduced-fat cheeses ( $\sim 5.5$  at 6%, w/w, fat *versus*  $\sim 5.2$ , at 6%, w/w, fat at 225 d), which would enhance the activity of the indigenous milk proteinase, plasmin (Grufferty and Fox, 1988). The increase in pH as the fat content increased may also affect the degree of hydration and aggregation of the  $\beta$ -casein (Creamer, 1985), which could affect its susceptibility to hydrolysis by chymosin or plasmin. In contrast to the trend noted for  $\beta$ -casein, the degradation of  $\alpha_{s1}$ -casein decreased as the fat content was reduced. This effect may be due to a number of associated factors:

- The decrease in the ratio of residual rennet activity-to-protein level (Fenelon and Guinee, 2000)
- The decrease in the level of MNFS (Table 11.1);
- The high pH of low-fat cheeses that is less favorable to the proteolytic activity of residual rennet (Tam and Whitaker, 1972; O'Keefe *et al.*, 1975).

### 11.6.2. Secondary Proteolysis

For Herrgård's and Drabant cheeses with similar MNFS, Ardö *et al.* (1993) found that the level of 5% phosphotungstic acid-soluble N (as % total N), which includes low molecular mass peptides ( $< 0.6$  kDa) and free amino acids (FAA) (Jarrett *et al.*, 1982), was scarcely affected by a 40% reduction in fat content. In contrast, reducing the level of fat in Feta-type (from  $\sim 22$  to 7%, w/w) or Kefalograviera-type (from  $\sim 31$  to 10%, w/w) cheeses lead to large decreases in the level of 5% phosphotungstic acid soluble N (as % total N, and as % of total cheese), despite similar levels of primary proteolysis in the corresponding full-fat and low-fat cheese types (Michaelidou *et al.*, 2003a, b). The discrepancy between the latter studies (Ardö *et al.*, 1993; Michaelidou *et al.*, 2003a, b) may be due to factors that affect the type of proteolysis, including differences in starter type, cheese making procedure, and cheese composition (Fox and Cogan, 2004; Lawrence *et al.*, 2004; McSweeney, 2004; Upadhyay *et al.*, 2004).

For cheeses with a different level of MNFS, the effect of reducing fat content on the degree of secondary proteolysis in Cheddar cheese is the

opposite to that noted for primary proteolysis (Fenelon *et al.*, 2000a). Reducing the level of fat led to a significant increase in the concentration of total FAAs per 100 g cheese (Figure 11.7b) but did not significantly affect the concentration of FFAs per 100 g cheese N. Hence, the increase in FAA as the fat content was reduced is due, at least partly, to the concomitant increase in the total concentration of protein in the cheese. Consistent with the higher level of FAAs in the reduced-fat cheeses, the level of low molecular mass peptides (< 1 kDa) in the pH 4.6-soluble cheese extracts increased as the fat content of the cheese decreased (Altemueller and Rosenberg, 1996; Fenelon *et al.*, 2000a).

### 11.7. Contribution of Lipolysis and Catabolism of Free Fatty Acids (FFA) to Cheese Flavor

Rennet-coagulated cheeses are ripened (matured) for a period from *ca.* two weeks (e.g., Mozzarella) to two or more years (e.g., Parmigiano-Reggiano or extra-mature Cheddar) during which time the flavor characteristic of the variety develops (McSweeney, 2004). Cheese generally contains several hundred volatile and non-volatile compounds at concentrations greater than their flavor thresholds. Hence, most workers now accept the “component balance” theory of cheese flavor (Mulder, 1952; Kosikowski and Moquot, 1958), which proposes that cheese flavor is caused by the correct balance and concentrations of a wide range of sapid compounds. Thus, differences between the flavors of different varieties are usually due to differences in the *balance and concentration* of flavor compounds rather than to the presence or absence of specific compounds. Indeed, most flavor compounds are present in most varieties but at very different concentrations.

The flavor compounds in cheese are produced during ripening by a complex series of microbiological changes and biochemical events. Biochemical transformations of residual lactose, and lactate and citrate, and proteolysis of the caseins to a large number of peptides and ultimately to free amino acids, which are subsequently catabolized to a range of volatile flavor compounds, are important in most cheeses (for recent reviews see McSweeney, 2004; McSweeney and Fox, 2004; Upadhyay *et al.*, 2004; Curtin and McSweeney, 2004). However, lipids in cheese also play important roles in the development of cheese flavor during ripening by:

- Affecting the rheology and texture of cheese and hence the rate of release of sapid compounds from the cheese matrix
- Acting as a source of fatty acids, which when liberated from triacylglycerols by the action of lipases, contribute directly to cheese flavor

(particularly in the case of short-chain FFAs) or indirectly, through their metabolism to a range of volatile flavor compounds, and

- Perhaps by facilitating reactions which occur at the fat-water interface (Collins *et al.*, 2004)

The role of milk-fat in the development of flavor in cheese during ripening will be discussed below although it should not be forgotten that lipolysis and the metabolism of fatty acids do not occur in isolation from other important biochemical events during ripening.

### 11.7.1. Lipolysis

In foods in general, fats undergo oxidative and hydrolytic degradation. However, since the oxidation-reduction potential of cheese is low (*ca.* –250 mV) and milk-fat contains low levels of polyunsaturated fatty acids, lipid oxidation does not occur to a significant extent in cheese during ripening (McSweeney and Sousa, 2000; Collins *et al.*, 2003a, 2004). However, the liberation of free fatty acids (FFA) by the action of lipases (lipolysis) is an important biochemical event during cheese ripening and has been studied extensively (see McSweeney and Sousa, 2000; Collins *et al.*, 2003a, 2004; McSweeney, 2004). Lipases in cheese originate from six possible sources:

- Milk
- Rennet paste
- Starter bacteria
- Secondary starter organisms (e.g., *Penicillium roqueforti*, *P. camemberti*, *Propionibacterium* sp., smear bacteria)
- Non-starter lactic acid bacteria (LAB)
- Exogenous addition

The indigenous lipase in bovine milk, lipoprotein lipase (LPL), is a 55 kDa homodimeric protein with catalytic optima at pH ~8.5 and 37°C. Under optimum conditions, there is sufficient LPL activity in milk to cause perceptible rancidity within *ca.* 10 s (Walstra and Jenness, 1984). However, in milk, enzyme and substrate are compartmentalized since *ca.* 90% of the enzyme is associated with the casein micelles and the fat is protected by the milk-fat globule membrane. LPL has a preference for medium-chain fatty acids and for acids esterified at the *sn*-1 or *sn*-3 position of triacylglycerol molecules at which are largely esterified short and medium chain fatty acids (Collins *et al.*, 2004). LPL activity is of most significance in cheese varieties made from raw milk since pasteurization largely inactivates this enzyme, although heating at 78°C × 15 s is needed for complete inactivation (Driessen, 1989).

Most commercial rennet preparations are free from lipase activity. However, the rennet pastes used for the manufacture of certain traditional Italian and Greek varieties (e.g., Provolone, the various Pecorino cheeses and traditional Feta) contain a potent lipase, pregastric esterase (PGE), the action of which contributes to the strong flavor of these cheeses (McSweeney and Sousa, 2000). PGE is produced by glands at the base of the tongue and is washed into the stomach with the milk on suckling. Rennet pastes are produced by macerating the abomasum of the young dairy animal into a paste, which is slurried in milk before use and they contain chymosin and pepsin in addition to PGE.

Although weakly lipolytic, starter and non-starter LAB are important sources of lipolytic enzymes in many varieties, particularly those without a strongly lipolytic secondary flora (e.g., Cheddar and Gouda) since LAB are present at high numbers for long periods of time. A number of lipolytic enzymes have been characterized from LAB most of which have optimal activity at *ca.* 37°C and pH 7.0–8.5. Most were assayed on soluble derivatives of fatty acids and were optimally active on substrates esterified to short-chain fatty acids and hence were esterases, although genuine lipase activity (assayed on emulsified substrates) has also been demonstrated in LAB (see Collins *et al.*, 2004). The lipolytic enzymes of LAB appear to be intracellular and thus must be released into the cheese matrix by cell lysis; the relationship between lipolysis and lysis was demonstrated in Cheddar cheese by Collins *et al.* (2003b).

The ripening of some varieties is characterized by the development of a secondary microflora, which often contributes greatly to lipolysis. In Blue cheese, *Penicillium roqueforti* grows in fissures in the cheese and produces two extracellular lipases with an optimum pH of 7.5–8 and 9–9.5, which cause extensive lipolysis in these varieties (McSweeney and Sousa, 2000). The white mold, *P. camemberti*, produces an extracellular lipase, which is optimally active at pH 9 and 35°C and which contributes to lipolysis in Camembert and Brie (Lamberet and Lenoir, 1976). Swiss cheeses are characterized by the growth of *Propionibacterium freudenreichii*, which is about 10–100 times more lipolytic than LAB (Dupuis, 1994) and produces an intracellular lipase with pH and temperature optima of 7.2 and 47°C, respectively (Oterholm *et al.*, 1970). Finally, smear-ripened cheeses initially develop the mold, *Geotrichum candidum*, on their surface followed by a complex Gram-positive bacterial flora. However, with the exception of *Brevibacterium linens* and *G. candidum*, lipases from these organisms have not been studied in detail (see Collins *et al.*, 2004). The surface flora contributes to the significant levels of lipolysis observed in these cheeses during ripening.

As shown for some examples in Table 11.4, the level of lipolysis in cheese varies considerable from moderate (e.g., Cheddar, Mozzarella and Edam) to very extensive (e.g., Blue cheese). Generally, cheeses that are characterized by a high level of FFAs are manufactured using rennet paste (e.g., Pecorino varieties), have a strongly lipolytic secondary microflora (e.g., Blue cheese or smear-ripened varieties) or have been ripened for a very long period (e.g., Parmigiano-Reggiano). High levels of FFAs in many varieties (e.g., Cheddar and Emmental) lead to cheeses being rejected as rancid (see Collins *et al.*, 2004). FFA, particularly short chain acids, contribute directly to cheese flavor but they also act as substrates for a range of catabolic reactions.

### 11.7.2. Metabolism of Fatty Acids

In addition to their direct role in cheese flavor, FFAs also act as precursors for a range of other volatile flavor compounds such as *n*-methyl ketones (alkan-2-ones), secondary alcohols, hydroxyacids, lactones, esters, and thioesters (Collins *et al.*, 2003a, 2004; McSweeney, 2004). General pathways through which FFAs are catabolised are shown in Figure 8.

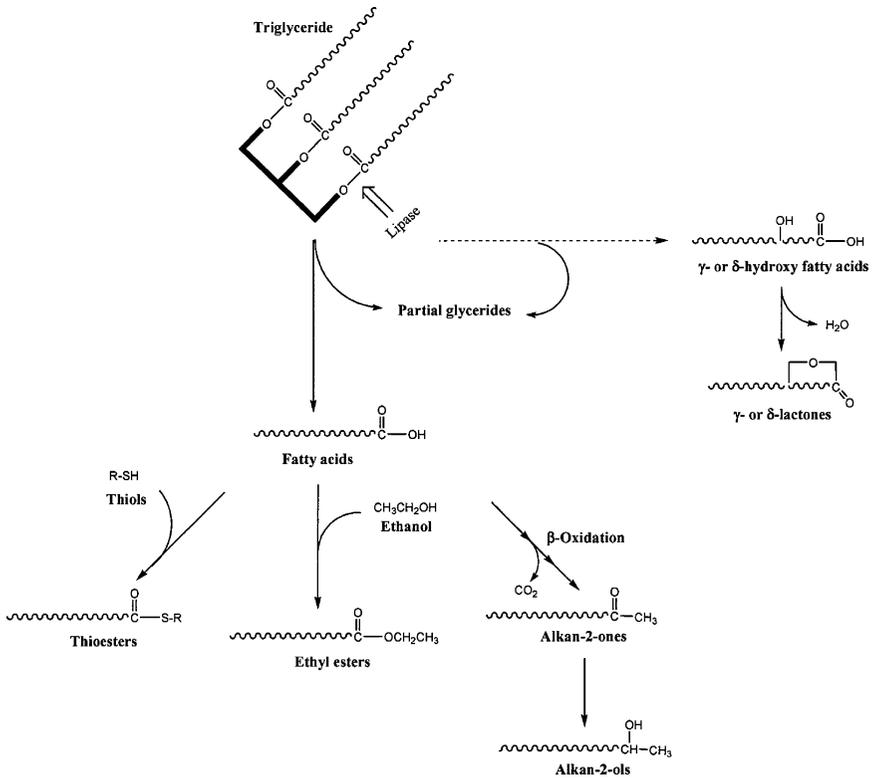
*n*-Methyl ketones (alkan-2-ones), particularly heptan-2-one and nonan-2-one, are the main compounds responsible for the characteristic pungent flavor of Blue cheese in which they are produced from fatty acids by the action of *P. roqueforti* through a pathway corresponding to the early stages of  $\beta$ -oxidation (Collins *et al.*, 2003a, 2004; McSweeney, 2004). The rate of production of methyl ketones in Blue cheese is affected by temperature, pH, physiological state of the mold and by the concentration of FFAs. Methyl ketones are also found in many other varieties at levels much lower than those found in Blue cheese (see Collins *et al.*, 2004, for references). Methyl ketones may be reduced to the corresponding secondary alcohol (see Figure 11.8).

Lactones are cyclic compounds formed through the intramolecular esterification of a hydroxy fatty acid.  $\gamma$ -Lactones and  $\delta$ -lactones, with five-sided and six-sided rings, respectively have been found in cheese (Jolly and Kosikowski, 1975; Wong *et al.*, 1975; Collins *et al.*, 2004). The origin of the precursor hydroxy fatty acids has been ascribed to a  $\delta$ -oxidation system in the mammary gland of ruminants (see Fox *et al.*, 2000), the reduction of keto acids (Wong *et al.*, 1975) and/or the action of lipoxigenases and other enzymes present in members of the rumen microflora (Dufossé *et al.*, 1994). Lactones have low flavor thresholds and while their aromas are not specifically cheese-like (their aromas have been described variously as “peach,” “apricot” and “coconut”), they may contribute to the overall flavor of cheese (see Collins *et al.*, 2004).

**Table 11.4.** Concentration (mg/kg Cheese) of Free Fatty Acids in Some Selected Cheese Varieties (From Mcsweeney, 2004, with Permission)

Cheese Type	C <sub>2:0</sub>	C <sub>4:0</sub>	C <sub>6:0</sub>	C <sub>8:0</sub>	C <sub>10:0</sub>	C <sub>12:0</sub>	C <sub>14:0</sub>	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	TOTAL	Reference
Parmesan	1055	451	243	440	439	1540	3896	1171	3471	123			13697	de la Feunte <i>et al.</i> (1993)
	476	952	143	175	159	571	952	1556	794	2841	635	238	9492	Kilcawley <i>et al.</i> (2001)
Cheddar	1587	952	191	159	175	619	746	1253	508	1476	413	175	8254	
	1270	794	111	111	48	238	397	619	270	667	206	111	4842	
Swiss	170	90	45	122	208	311	1904	1427*					4277	Woo <i>et al.</i> (1984)
	345	21	25	53	88	267	930	1197*					2926	
Edam	60	8	9	14	47	39	122	57*					356	Woo <i>et al.</i> (1984)
Mozzarella	54	7	1	120	12	27	76	66*						Woo and Lindsay, 1984
Provolone	782	308	81	172	122	120	199	334*						
Camembert	35	5	14	35	43	69	270	210*					681	Woo <i>et al.</i> , 1984
Camembert	101	58				448	1028		1421					Lesage <i>et al.</i> (1993)
Camembert	361	287	160	225	298	622	1442	303	1043				5066	de la Feunte <i>et al.</i> (1993)
Roquefort	961	626	707	2280	1295	3185	6230	2241	6282	896			25969	de la Feunte <i>et al.</i> (1993)
Port Salut	41	4	8	54	33	86	275	199*					700	Woo <i>et al.</i> (1984)
Limburger	1475	688	24	50	92	602	565	709*						
Münster	163	102	66	154	206	704	2057	833	1412	59	504			de Leon-Gonzalez <i>et al.</i> (2000)

\*C18:0 congeners



**Figure 11.8.** Pathways for the catabolism of free fatty acids in cheese during ripening (reprinted from *Cheese: Chemistry, Physics and Microbiology*, 3rd edn P.F. Fox *et al.* (eds.), Collins, Y.F., McSweeney, P.L.H., Wilkinson, M.G., Lipolysis and Catabolism of fatty acids in cheese, pp. 373–379, 2004, with permission from Elsevier).

Esters of short to medium-chain FFAs are commonly found in many cheese varieties (Meinhart and Schreier, 1986; Gonzalez de Llano *et al.*, 1990; Imhof and Bosset, 1994; Arora *et al.*, 1995; McSweeney, 2004). FFAs are most commonly esterified to ethanol, although low levels of methyl esters have also been found in cheese (e.g., Villaseñor *et al.*, 2000). Ethanol that may be produced from lactose metabolism or through the catabolism of FFAs, is usually the limiting reactant in the production of esters (Collins *et al.*, 2004). Although ethyl esters may be produced by direct reaction of an FFA with ethanol, Holland *et al.* (2002) suggested that they may arise during cheese ripening by the transesterification of an FFA from a partial glyceride to ethanol. Most esters have a buttery or fruity aroma

(Arora *et al.*, 1995; Engels *et al.*, 1997). Thioesters are formed by the reaction of an FFA with a sulphhydryl compound, particularly methanethiol ( $\text{CH}_3\text{SH}$ ; Molimard and Spinnler, 1996; Collins *et al.*, 2003a, 2004). Methylthioesters of short-chain FFAs have been associated with the characteristic aromas of Cheddar and smear-ripened cheeses (Arora *et al.*, 1995; Lamberet *et al.*, 1997).

### 11.8. Effect of Fat on the Fracture-Related Properties of Unheated Cheese

In most applications, whether as a consumer product or as an ingredient, cheese is subjected to size-reduction operations. Cheese may be portioned (e.g., for consumer packs), sliced, crumbled into irregularly-shaped pieces (e.g., Feta or Stilton for salads), shredded into cylindrical pieces (e.g., 2.5 cm long and 0.4 cm diameter; for sandwiches, pizza), diced into very small cubes (e.g., 0.4 cm, for salads) grated into particles  $< 1$  mm (e.g., for dried parmesan), or comminuted by forcing pre-cut cheese through die plates with narrow apertures (e.g., in preparation of sauces or processed cheese products). Similarly, when eaten, cheese is subjected to a number of forces, which reduce it to a paste before being swallowed; first, the cheese is bitten (cut by the incisors), compressed (by the molars) on chewing, and sheared (between the palate and the tongue, and between the teeth). During the above applications the cheese is subjected to high stresses (e.g.,  $> 200$  kPa) and strains (e.g.,  $> 70\%$ ), which result in fracture of the cheese mass to varying degrees. The behavior of the cheese when exposed to the different size-reduction methods constitutes a group of important functional attributes, including shreddability, sliceability, gratability, spreadability, hardness, and crumbliness (Guinee and Kilcawley, 2004). These attributes are determined largely by the rheological characteristics of the cheese, which define its deformation and/or flow when subjected to a stress and/or strain (O'Callaghan and Guinee, 2004).

Large-strain deformation testing, using uniaxial compression on a texture analyzer, may be used easily to apply strains in the range encountered during size-reduction operations. The cheese may be subjected to 1 or 2 compression cycles (Texture Profile Analysis; TPA). Large-strain compression is most commonly used to assess the rheological properties of cheese. Several rheological quantities, which may be related to the functional attributes of the cheese, can be obtained from the resultant stress strain/curve (O'Callaghan and Guinee, 2004). These include fracture stress ( $\sigma_f$ ), fracture strain ( $\varepsilon_f$ ), firmness ( $\sigma_{\max}$ ), cohesiveness, gumminess, chewiness, and adhesiveness. Other tests (large deformation shear, wire cutting, and bending)

may also be used to assess the large-strain deformation properties (O'Callaghan and Guinee, 2004).

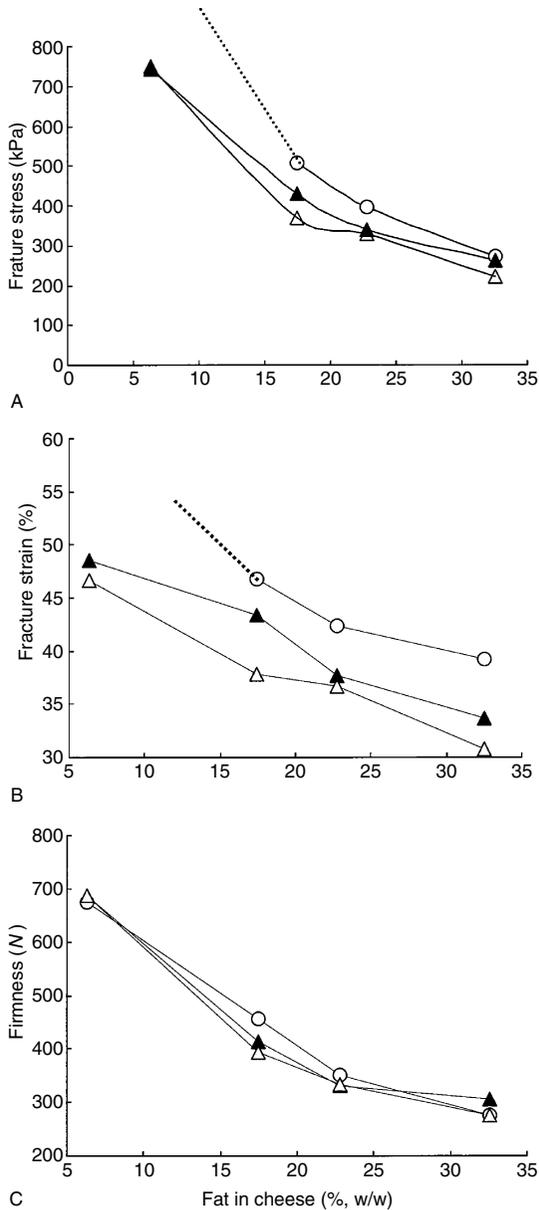
### 11.8.1. Effect of Fat Content on Fracture Properties

Altering the fat content has marked effects on the fracture-related properties of different cheese varieties, including Cheddar (Emmons *et al.*, 1980; Bryant *et al.*, 1995; Mackey and Desai, 1995; Fenelon and Guinee, 2000; Gwartney *et al.*, 2002), Mozzarella (Tunick *et al.*, 1991, 1993a, b, 1995; Tunick and Shieh, 1995) and Cottage cheese (Rosenberg *et al.*, 1995). Reducing the level of fat in Cheddar cheese results in increases in elasticity,  $\sigma_f$ ,  $\varepsilon_f$ ,  $\sigma_{\max}$  (Figure 11.9), cohesiveness, springiness, chewiness, and gumminess and a decrease in adhesiveness; consequently, the texture of reduced-fat Cheddar tends to be much less acceptable to the consumer than that of full-fat Cheddar, which is much softer and less rubbery and tough. The adverse effects of fat reduction are expected because of the concomitant increase in the concentration of intact casein and its contribution to cheese elasticity (see Guinee, 2003; Guinee and Kilcawley, 2004). Moreover, liquid fat acts as a lubricant on fracture surfaces of the casein matrix, and reduction of the fat level is thereby expected to increase the stress required to fracture the matrix (Marshall, 1990; Prentice *et al.*, 1993). Thus, while Chen *et al.* (1979) found that the hardness of different cheese varieties generally tended to increase as the level of fat was reduced, the relationship between fat and hardness was not significant.

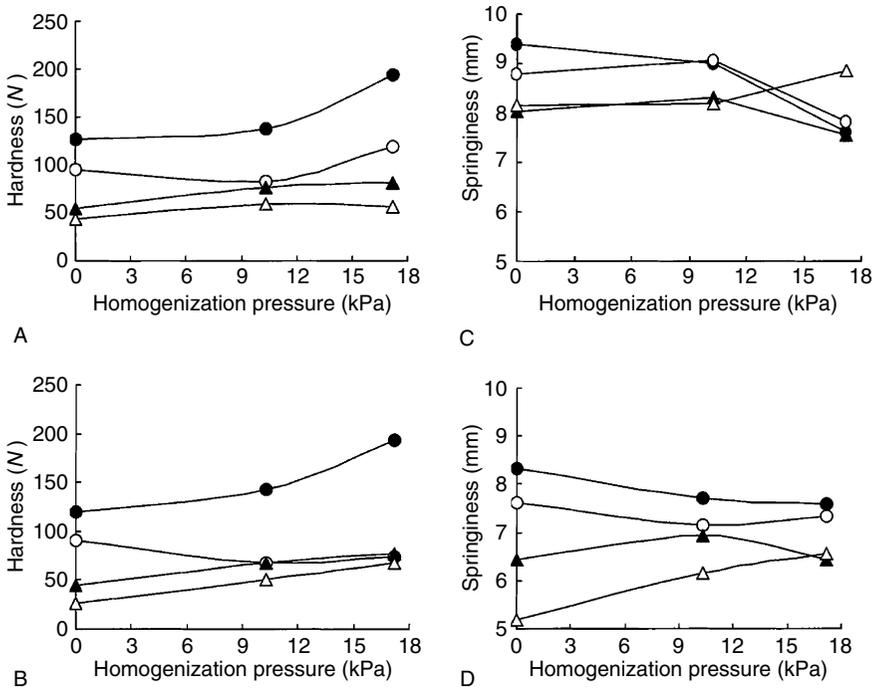
Similar trends have been noted for the effect of fat content on the rheological properties of Mozzarella cheese (Tunick *et al.*, 1993a, b; Tunick and Shieh, 1995). Reducing the fat content (e.g., 21–25%, w/w, to ~9–11%, w/w) of low- and high-moisture Mozzarella cheeses (47.7–51.8%, w/w, and 52.2–57.4%, w/w, respectively) resulted in significant increases in hardness and springiness, with the magnitude of the effect being most pronounced for hardness (Figure 11.10). There was a significant effect of the interaction between scald temperature and fat content on hardness, with the effect of fat reduction on hardness being more pronounced as the scald temperature was raised from 32.4°C to 45.9°C.

### 11.8.2. Effect of Solid-to-Liquid Fat Ratio on Fracture Properties

Increasing the liquid-to-solid fat ratio, by raising the temperature of the cheese in the range 0–40°C, results in large decreases in the  $\sigma_f$  and  $\sigma_{\max}$  of different cheese types including Gouda (Culioli and Sherman, 1976), Cheshire and Leicester (Dickinson and Goulding, 1980), and 4 week-old Brie (Molander *et al.*, 1990). However, in these studies, the change in  $\varepsilon_f$  on increasing temperature depended on cheese type: it did not change with



**Figure 11.9.** Effect of fat content on fracture stress (A), fracture strain (B) and firmness (C) of Cheddar cheeses aged for 120 (○), 180 (▲) or 225 (△) days. Broken line indicates that the sample did not fracture on compression at the early ripening times (drawn from data of Fenelon and Guinee, 1999; Guinee *et al.*, 2000a).



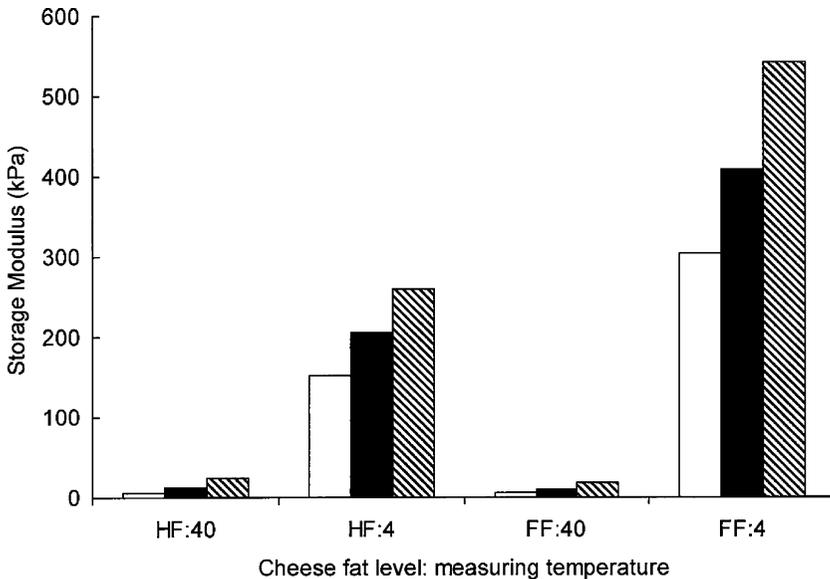
**Figure 11.10.** Effect of homogenization pressure on the hardness (A, B) and springiness (C, D) of 1 week (a, c) and 6 week (b, d)-old Mozzarella cheeses with different levels of fat-in-dry matter (low-fat, LF, ~22%, w/w; high-fat, HF, ~49%, w/w) and manufactured using a low (LT, 32°C) or high (HT, 45.9°C) curd scalding temperature: LFHT (●), LFLT (○), HFHT (▲), or HFLT (△) (drawn from data of Tunick *et al.*, 1993b).

temperature (0–40°C) for Leicester, increased slightly for 4 week-old Brie (5–20°C), increased by a factor of 2 for Cheshire over temperature range (0–40°C), and decreased slightly for Gouda (10–20°C).

The changes in  $\sigma_f$  and  $\sigma_{max}$  are expected as milk fat, which is predominantly solid at 0°C, is almost entirely liquid at 40°C (Wright *et al.*, 2002). Hence, at a low temperature (e.g., 4°C), the fat globules encased within the *para*-casein network are essentially solid and augment the elastic contribution of the casein matrix and increase the stress required to achieve a given deformation or fracture; deformation of the casein matrix would also require deformation of the fat globules enmeshed within its pores. However, the contribution of fat to cheese elasticity decreases as the ratio of solid-to-liquid fat decreases with increasing temperature, and is very low at 40°C. At the higher temperatures, the fat behaves more as a fluid and the fat globules

confer viscosity rather than elasticity or rigidity on the cheese mass. Hence, on the application of a stress at 40°C, the fat globules flow to an extent dependent, *inter alia*, on the liquid-to-solid fat ratio and the strength of the casein matrix, which determines the degree of deformation of the matrix itself and its occluded fat globules.

This effect of temperature is readily apparent from the sharp decrease in elastic shear modulus,  $G'$ , and increase in phase angle,  $\delta$ , as measured using low-strain (e.g., 0.006) oscillatory rheometry, when cheese is heated from 20 to 40°C (see Section 11.9). The effect of temperature on the rheological properties is also observed by comparing the effect of temperature on the  $G'$  of cheeses differing in fat content. The magnitude of the  $G'$  for half-fat Cheddar is lower than that of full-fat Cheddar at 4°C when the fat is mainly solid but similar at 40°C when fat is mainly liquid, even though the dry matter content of the latter is higher than that of the former (Figure 11.11). Other studies on Cheddar cheese have also shown that  $G'$  at 40°C decreased as the fat content was increased in the range 1.3–34%, w/w (Ustanol *et al.*, 1995; Guinee *et al.*, 2000c). In contrast to the foregoing, Ma *et al.* (1997) reported that  $G'$  for full-fat Cheddar at 20°C was higher



**Figure 11.11.** Effect of fat level (half-fat, HF, 17%, w/w; full-fat, FF, 32%, w/w) and assay temperature (4 or 40°C) on the elastic shear modulus of Cheddar cheeses measured using low-amplitude strain oscillation at a frequency of  $\sim 0.1$  (□), 1 (■) or 10 (▨), Hz.

than that of half-fat Cheddar. From a sensory viewpoint, it may be assumed that cheese rapidly approaches body temperature, (i.e.,  $\sim 37^{\circ}\text{C}$ ), on ingestion. At this temperature, the cheese fat is almost fully liquid (Wright *et al.*, 2002) and therefore confers fluidity and lubrication to the cheese, and mouth coating, on mastication.

### 11.8.3. Effect of Homogenization of Milk or Cream, and Degree of Fat Emulsification on Fracture Properties

Homogenization of cheese milk or cream reduces the hardness and fracture stress of reduced-fat Cheddar (Emmons *et al.*, 1980; Jana and Upadhyay, 1993; Metzger and Mistry, 1994) and other varieties (Jana and Upadhyay, 1991, 1992). Because of these effects, homogenization improves the textural quality of reduced-fat Cheddar cheese (Metzger and Mistry, 1994). The positive effects of homogenization coincide with higher levels of MNFS and moisture, which has been found to reduce  $E$ ,  $\sigma_f$  and/or  $\sigma_{\max}$  and increase  $\varepsilon_f$  and adhesiveness in different cheese types, including Dutch-type Meshanger (de Jong, 1978a), Gouda (Luyten, 1988; Visser, 1991), and experimental Cheddar-type cheeses (Watkinson *et al.*, 2002).

Jana and Upadhyay (1991, 1993) reported that homogenization of milk for full-fat Mozzarella ( $\sim 22\%$  w/w fat) cheese, at a combined first and second stage pressure of 250 or 500 kPa, significantly reduced hardness and springiness, and increased cohesiveness; simultaneously, there were non-significant decreases in gumminess and chewiness. The magnitude of the effect, which increased with homogenization pressure, coincided with a decrease in protein content and increases in the levels of moisture and MNFS. In contrast, studies on reduced-fat Mozzarella (8–13%, w/w, fat) showed that homogenization of cheese milk or cream [first and second stage pressures of 13.8 and 3.45 MPa (Rudan *et al.*, 1998b) or 17.3 and 3.4 MPa, (Poduval and Mistry, 1999)] did not significantly affect the hardness or springiness of reduced-fat ( $\sim 8\%$  w/w) Mozzarella cheese at 30 d. An opposite trend to the above was reported by Tunick *et al.* (1995); two-stage homogenization of milk, at a combined first and second stage pressure of 10.3 or 17.2 MPa, resulted in a general increase in the hardness (at  $23^{\circ}\text{C}$ ) of low-fat or high-fat Mozzarella after storage for 1–6 wk (Figure 11.10). There was a significant effect of the interaction between homogenization pressure and scald temperature used in cheese manufacture, with the increase in hardness being more pronounced in the cheese scalded at the higher temperature (Figure 11.10a, b). The magnitude of the effect also tended to be more pronounced at the lower fat content. Likewise, there was significant interaction between fat content and homogenization pressure on springiness (Figure 11.10 c, d) (Tunick *et al.*, 1995); the increase in springiness as the

fat content was reduced tended to diminish with increasing homogenization pressure.

Discrepancies between the above studies *vis-à-vis* the effects of homogenization on the textural and rheological characteristics of cheese may be due to differences in homogenization conditions, assay conditions, age of cheese, and fat content.

## 11.9. Effect of Fat on the Functional Properties of Heated Cheese

As an ingredient, cheese is used extensively in cooking applications (e.g., grilled cheese sandwiches, pizza pie, cheeseburgers, pasta dishes, and sauces) in which it reaches a temperature of  $\sim 80\text{--}100^\circ\text{C}$ . A key aspect of the cooking performance of cheese is its heat-induced functionality, which is a composite of different attributes. Depending on the application, one or more functional attributes may be required. The various functional properties of heated cheese and their interpretation have been reviewed by Kindstedt *et al.* (2004) and Guinee and Kilcawley (2004). Some of the main functional attributes include meltability (softening), stretchability, flowability (or spreadability), apparent viscosity, succulence, sheen, and/or tendency to brown. Flowability, a measure of the ability of cheese to spread during heating, and stretchability, a measure of the ability of the heated cheese to form strings and/or sheets when extended, involve strain displacement as a result of stresses on the *para*-casein matrix. Stress may occur spontaneously during quiescent heating (e.g., baking), as a result of collapse, flow and coalescence of melted fat globules/pools, which when solid (e.g., at  $4^\circ\text{C}$ ) contribute to rigidity and physical support of the matrix. Conversely, an external stress may be applied to the hot molten cheese mass after cooking, (e.g., during consumption or instrumentally during testing; shear during viscometric testing, compression during squeeze flow evaluation, or extension during stretchability testing).

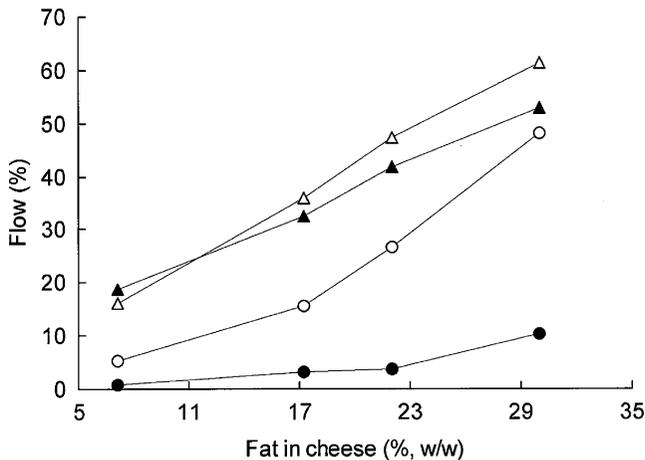
### 11.9.1. Effect of Fat Level on Cooking Properties

The effects of fat reduction on the heat-induced functionality of cheeses, especially Mozzarella and Cheddar, has been investigated extensively and reviewed in the last decade (Tunick *et al.*, 1993a, b; Merrill *et al.*, 1994; Fife *et al.*, 1996; McMahon *et al.*, 1996; Rudan and Barbano, 1998; Poudaval and Mistry, 1999; Rudan *et al.*, 1999; Guinee *et al.*, 2000a; Metzger *et al.*, 2001a, b; Sheehan and Guinee, 2004; Kindstedt *et al.*, 2004; Guinee and Kilcawley, 2004). The concentration on Cheddar and Mozzarella reflects their economic importance (together, they account for  $\sim 30\%$  of

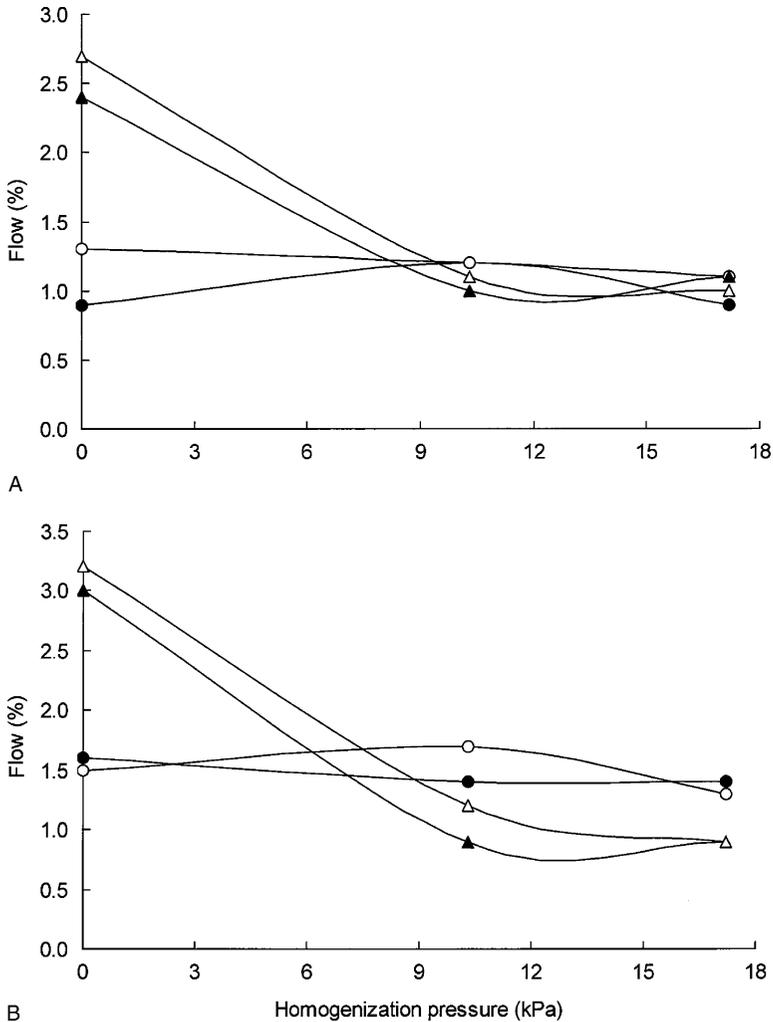
total cheese produced globally) and their extensive use as ingredient in cooked foods.

Reducing the fat content impairs the functionality of Cheddar and Mozzarella, as reflected by decreases in flowability and stretchability and an increase in the apparent viscosity of the melted cheese (Figures 11.12, 13, 14). The flowability of 1 and 6 wk-old Mozzarella decreased by 9–25% as the fat content was reduced from ~25–9% (w/w) (Tunick *et al.*, 1993b, 1995; Tunick and Shieh, 1995), while the flowability of Cheddar cheeses ripened for  $\leq 180$  d decreased by 70 to 95% on reducing the fat level from 32 to 6% (w/w) (Guinee *et al.*, 2000a).

Olson and Bogenrief (1995) noted that the difference in flowability between full-fat and reduced-fat Cheddar cheese (with 50 or 75% of fat content in full-fat cheese) decreased with ripening time and had disappeared after 200 d storage at 7°C, when the cheese was melted for 5 min. In contrast, Guinee *et al.* (2000 a, c) reported that the differences in flowability between Cheddar cheeses with a fat content ranging from 6 to 32%, w/w, persisted throughout an 180 day ripening period; moreover, the negative relationship between flow and fat level was essentially linear at all ripening times investigated (Figure 11.12). The discrepancy between the foregoing studies may be due to differences in the measurement technique, which affected the propensity of the cheese to dehydrate during cooking. Olson and Bogenrief (1995) used a cylinder of cheese in a Pyrex glass tube in a water bath at 94°C,



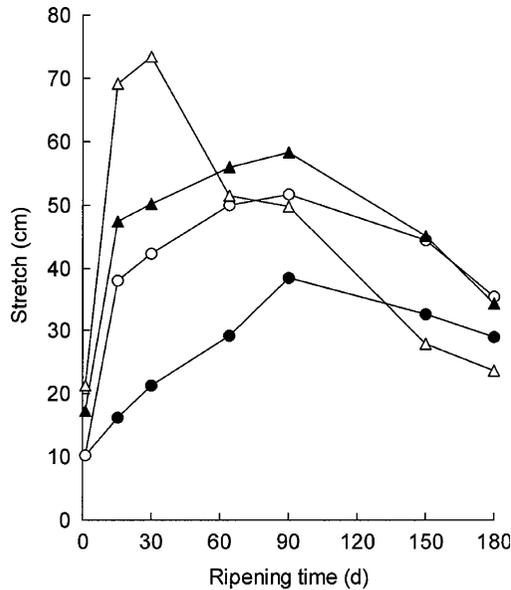
**Figure 11.12.** Effect of fat content on the flowability of Cheddar cheeses aged for 1 (●), 30 (○), 90 (▲), or 180 (△) days, on baking at 280°C for 4 min (redrawn from data of Guinee *et al.*, 2000a).



**Figure 11.13.** Effect of homogenization pressure on the flowability of 1 week (A) and 6 week (B) -old Mozzarella cheeses with different levels of fat-in-dry matter (low fat, LF, ~22%, w/w; high fat, HF, ~49%, w/w) and manufactured using a low (LT, 32°C) or high (HT, 45.9°C) curd scalding temperature: LFHT (●), LFLT (○), HFHT (▲), or HFLT (△) (redrawn from data from Tunick *et al.*, 1993b).

while Guinee *et al.* (2000a,c) used a disc of cheese placed on a stainless steel surface in a convection oven at 280°C for 4 min.

The adverse effects of fat reduction on flowability may be attributed to a number of factors, including the increased level of intact casein, and



**Figure 11.14.** Age-related changes in the stretchability of Cheddar cheeses of different fat content on baking at 280°C for 4 min: low-fat (7.2%, w/w; ●), half-fat (17.2%, w/w; ○), reduced-fat (21.9%, w/w; ▲) and full-fat (30.5%, w/w; △) (redrawn from data of Guinee *et al.*, 2000a).

reduced levels of proteolysis, moisture-to-protein ratio and heat-induced fat coalescence. The net impact of these changes, which are discussed below, is a reduction in the level of displacement of adjoining layers of the casein matrix for a given level of stress.

Large reductions in fat content are paralleled by increases in the volume fraction of the casein matrix and level of intact casein in the unheated cheese (Figures 11.1, 2). The increase in intact casein is positively correlated with the apparent viscosity, and negatively with flowability, of the heated cheese (Guinee *et al.*, 2000a). The concomitant reduction in the number of fat globules is conducive to a higher degree of aggregation and fusion of casein strands during gel formation (McMahon *et al.*, 1993). Fat in the gel exists as globules occluded within the casein network, which physically impede casein aggregation. The higher degree of casein aggregation is unfavorable to heat-induced flow due to:

- A probable decrease in the degree of heat-induced slippage of contiguous casein layers
- A reduction in the content of MNFS (Table 11.1, Figure 11.1)
- A lower degree of casein hydration in the resultant cheese

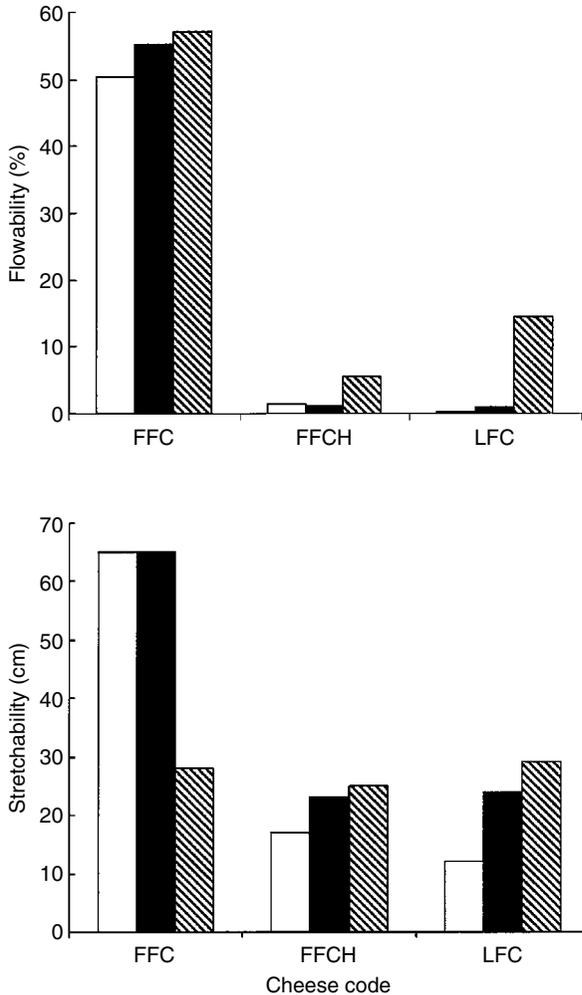
The low degree of casein hydration is less favorable to moisture retention during baking and results in dehydration, crusting and poor flow (Kindstedt, 1995; Kindstedt and Guo, 1997). Moisture also acts as a lubricant between the protein layers and between protein and fat layers during melting and thereby facilitates heat-induced slippage of different parts of the matrix (McMahon *et al.*, 1993; Prentice *et al.*, 1993).

The level of primary proteolysis in cheese decreases as the level of fat is lowered (Section 11.6). However, several studies have shown a positive relationship between the level of proteolysis and flowability in cheeses with a fixed fat content, [e.g., Mozzarella (Yun *et al.*, 1993a, b; Madsen and Qvist, 1998), Cheddar (Arnott *et al.*, 1957; Bogenrief and Olson, 1995) and model acid-curd cheeses (Lazaridis *et al.*, 1981)]. The positive effect of proteolysis on flowability may be due to a number of concomitant changes, including the increased water-binding capacity (Kindstedt, 1995), and an increase in the number of discontinuities or 'breaks' in the *para*-casein matrix at the micro-structural level (de Jong, 1978a). The latter factors are expected to promote a decrease in casein aggregation, an occurrence that should enhance heat-induced displacement of adjoining layers of the casein matrix.

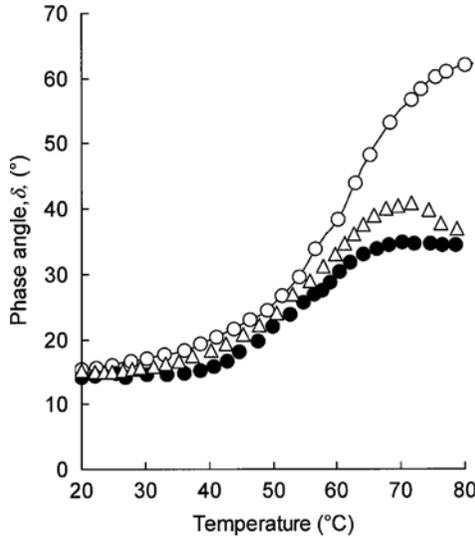
A reduction in fat level results in a decrease in the moisture-to-protein ratio, as reflected by the lower content of MNFS (Figure 11.1; Table 11.1). However, the flowability of rennet-curd cheeses is positively correlated with the content of MNFS (Rüegg *et al.*, 1991; McMahon *et al.*, 1993), an effect which may, in part, be due to the concomitant increase in casein hydration and the lubrication effect of moisture.

The degree of fat globule clumping and coalescence in both the unheated and heated (to 90°C) Cheddar cheese decrease as the fat level is reduced (Section 11.3). Coalescence of fat results in an increase in the continuity of the fat phase and in the free oil (FO) on heating the cheese. FO forms a layer on the surface of melting cheese, which limits the evaporation of moisture during cooking and the occurrence of associated defects such as skin formation, scorching, and impaired flow and stretch (Rudan and Barbano, 1998). Moreover, FO lubricates the displacement of adjoining layers of the casein matrix and thereby contributes positively to flow and stretch. An increase in FO may also alter the polarity of the solvent system (water and oil) in contact with the *para*-casein matrix and thereby increase the degree of solvation of the *para*-casein *per se*. A study by Hokes *et al.* (1982) on model analogue cheese systems showed that the addition of water to a dispersion of calcium caseinate in heated vegetable oil (or other polar solvents such as dioxane) resulted in gelation of the calcium caseinate. The latter effect was attributed to an increase in the polarity of the solvent system. Considering this, it is conceivable that free oil formed on heating cheese may be conducive to a structural rearrangement (e.g., exposure of

hydrophobic groups), and increase in level of solvation of the *para*-casein molecules. An increase in casein solvation is, in turn, expected to favor an increase in the heat-induced fluidity and flowability of the cheese. Hence, it is noteworthy that:



**Figure 11.15.** Effect of homogenization pressure on the flowability and stretchability of full-fat Cheddar-type cheese prepared from non-homogenized control milk (FFC) or control milk homogenized at respective first and second stages pressures of 25 and 5 Pa (FFCH), and of low fat Cheddar-type cheese from non-homogenized skimmed milk (LFC), after storage at 7°C for 5 (□), 70 (■) or 156 (▨) days (redrawn from data of Guinee *et al.*, 2000c).



**Figure 11.16.** Phase angle,  $\delta$ , as a function of temperature for 5 day-old Cheddar type cheese of different fat content, from non-homogenized ( $\Delta$ ,  $\circ$ ) or homogenized ( $\bullet$ ) milks. The cheeses were low-fat (1.3%, w/w;  $\Delta$ ); full-fat (30.0%, w/w;  $\circ$ ), and full-fat homogenized (30.6%, w/w;  $\bullet$ ) (redrawn from data of Guinee *et al.*, 2000c).

- The flowability of melted Cheddar and Mozzarella increases with fat content (Figures 11.12, 13) and
- The increase in phase angle, and thus fluidity, on heating low-fat Cheddar cheese or full-fat Cheddar cheese made from homogenized milk are much lower than that on heating full-fat Cheddar cheese made from non-homogenized milk (Figure 11.16).

### 11.9.2. Effect of Milk Homogenization and Degree of Fat Emulsification

Increasing the degree of fat emulsification (DE) in cheese by homogenization of the cheese milk (e.g., at first and second stage pressures of 25 and 5 MPa) reduces the flowability, stretchability and fluidity of heated full-fat Cheddar (Figure 11.15; Guinee *et al.*, 2000a, c). The effect of homogenization, which for Cheddar is similar to reducing its fat content from 30 to 1.3%, w/w (Figure 11.15), would be highly undesirable in applications such as pizza but highly desirable where a high degree of flow resistance is required, (e.g., in frying). Similarly, increasing the homogenization pressure of milk, in the range 0.4–6.7 MPa, leads to a progressive deterioration in the

flowability and stretchability of Halloumi cheese prepared from fresh milk or from reconstituted low-heat skim milk powder (RSMP) and anhydrous milk fat (AMF) (Lelievre *et al.*, 1990). However, the adverse effects of homogenization at the high pressure (6.7 MPa) on the melt characteristics of Halloumi were reversed by the addition of lecithin to the RSMP/AMF during the preparation of the recombined milk. Homogenization of cheese milk, or the cream used to standardize cheese milk, has also been found to impair flowability and reduce the level of free oil released from Mozzarella cheeses with a fat content of  $\sim 19\text{--}25\%$  (w/w) on baking, with the effect generally becoming more pronounced as the homogenization pressure is increased (Jana and Upadhyay, 1992; Tunick *et al.*, 1993b; Rowney *et al.*, 2003). In contrast, homogenization of milk or cream at similar pressures does not adversely affect the flowability or apparent viscosity (AV) of reduced-fat Mozzarella ( $\leq \sim 11\%$ , w/w, fat), but leads to a significant reduction in the content of free oil (FO) released on baking (Rudan *et al.*, 1998b; Poduval and Mistry, 1999). The similarity in flowability and AV for low-fat cheeses made from homogenized, or unhomogenized, milks despite the differences in FO was probably due to the very low level of FO in all cheeses; FO as a percentage total fat in cheese was  $\sim 0.5$ ,  $0.5$  and  $3.9$  for the homogenized milk cheese, homogenized cream cheese and the control at 40 d. Using the same analytical methodology, the FO values for commercial low-moisture part-skim Mozzarella ranged from  $\sim 10$  to  $40\%$  of total fat in cheese, depending on the FDM and age (Kindstedt and Rippe, 1990; Kindstedt, 1993). Thus, it is noteworthy that Tunick *et al.* (1993b) reported that the interaction between fat content and homogenization pressure had a significant effect on the flowability of 1 or 6 week-old Mozzarella (Figure 11.13). Homogenization of milk had little effect on the melt properties of low-fat Mozzarella ( $\sim 10\%$ , w/w, fat) but markedly impaired those of Mozzarella with a higher fat level ( $\sim 25\%$ , w/w).

Increasing the degree of emulsification of fat in pasteurized processed, and analogue, cheese products (by selective use of emulsifying salts and extending the duration of processing) also leads to a marked reduction in flowability (Rayan *et al.*, 1980) and loss of fluidity, as reflected by a decrease in the loss tangent ( $\tan \delta$ ) at  $80^\circ\text{C}$  (Neville, 1998).

The adverse effects of increasing the degree of emulsification on the functional properties of heated cheese probably ensue from the combined effects of:

- The interaction of *para*-casein-covered emulsified fat particles (which may be considered as pseudo-protein particles) with the casein matrix of the cheese, and
- A lower degree of heat-induced fat coalescence on melting the cheese.

On homogenization of milk, the native fat globule membrane is largely displaced and replaced by an adsorbed layer comprised of casein micelles, sub-micelles and whey proteins (Walstra and Jenness, 1984; Keenan, 1988). The newly formed recombined fat globule membrane interacts with the “free” micelles and thereby enables the emulsified fat particles to become an integral part of the matrix formed during cheese making, rather than being occluded within the matrix as inert particles (van Vliet and Dentener-Kikkert, 1982; Walstra and van Vliet, 1986). The incorporation of the emulsified fat particles into the casein matrix increases the effective protein concentration and the overall level of protein-protein interactions. Consequently, it is expected that functional properties relying on displacement of contiguous layers of the casein matrix (e.g., flow or stretch) would be impaired by the homogenization of cheese milk. Moreover, the recombined fat globule membrane stabilizes the newly formed fat globules to heat-induced coalescence (Guinee *et al.*, 2000c), as reflected by the general reduction in free oil when the cheese milk, or cream used for standardization of the cheese milk, is homogenized at combined first and second stage pressures of 0.5–20 MPa (Lelievre *et al.*, 1990; Jana and Upadhyay, 1991, 1993; Metzger and Mistry, 1995; Poduval and Mistry, 1999; Rowney *et al.*, 2003). The consequent reduction in FO predisposes the cheese to dehydration during heating (Rudan and Barbano, 1998) and reduces the lubricating effect of oil on the surfaces of adjoining layers of the *para*-casein matrix during displacement. Thus, the adverse effects of homogenization on flowability and stretchability may be reduced by (Lelievre *et al.*, 1990):

- Lowering the homogenization pressure that has the effect of reducing the surface area of the fat phase and the number of newly formed fat globules;
- Preventing the casein micelles from adsorbing at the fat-water interface by using a surface film of lecithin that has the effect of making the newly formed globules more susceptible to heat-induced coalescence.

### 11.9.3. Effect of Milk Fat Fraction on Cooking Properties

Rowney *et al.* (2003) compared the effect of anhydrous milk fat (AMF, melting point 29.5°C) and fat fractions of different melting points, obtained from the AMF and dispersed into the cheese milk at a low homogenization pressure (2.6 MPa at 50°C), on the functionality of Mozzarella cheese. The fractions and their melting points were: olein, (27°C) and stearin (43.2°C). Increasing the melting point led to a significant increase in the level of free oil in the cheese and a reduction in the viscosity of the heated (60°C) 3 d-old

cheese. The results suggest the higher fluidity of the low melting point fraction is conducive to the movement of the non-globular fat (formed during cheese manufacture) throughout the cheese mass during storage and its coalescence (see Section 11.3).

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## Ice Cream

H.D. Goff

### 12.1. Overview of Ice Cream Ingredients and Manufacture

Fat and fat structure development in ice cream and related frozen dairy desserts are critical for optimal structure and physical properties, stability, flavor and texture. This chapter will present a brief review of the functionality of fat in ice cream, with citations of the most recent and most pertinent references. In particular, readers are referred to Berger (1997), Goff (1997, 2002, 2003), Buchheim (1998), Marshall *et al.* (2003) and Goff and Tharp (2004) for more detailed information.

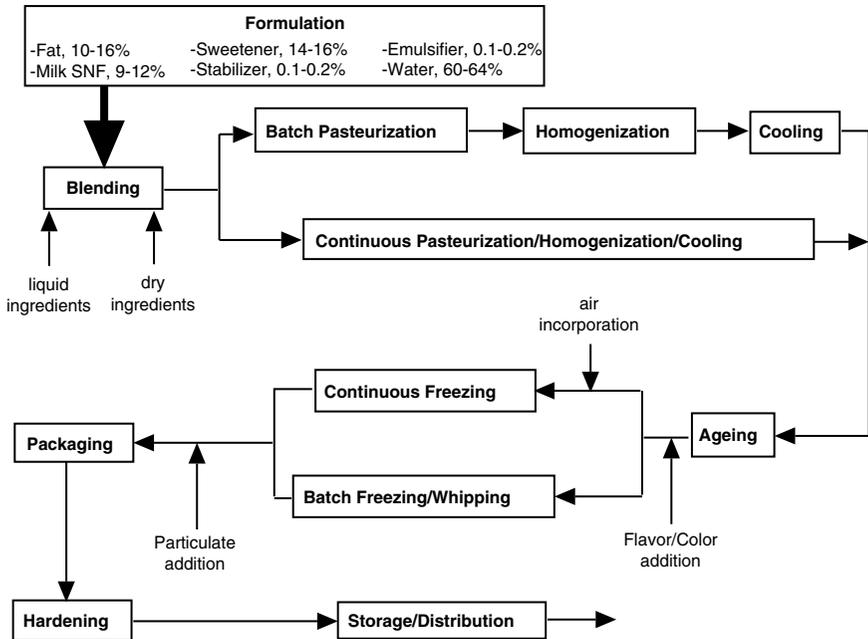
The term “ice cream” in its generic sense includes all whipped dairy products that are manufactured by freezing and are consumed in the frozen state, including ice cream that contains either dairy or non-dairy fats, premium (higher-fat) versions, “light” (lower-fat) versions, ice milk, sherbet and frozen yogurt. Ice cream mix formulations specify the content of fat, milk solids-not-fat, sweeteners, stabilizers, emulsifiers and water that are desired (Figure 12.1). Dairy and other ingredients used to supply these components are chosen on the basis of availability, cost, legislation, and desired quality. Common ingredients include: cream, butter or vegetable fats, as the main sources of fat; condensed skim or whole milk, skim milk powder, and/or whey powder or whey protein products, as the sources of concentrated milk solids-not-fat; sucrose and corn starch hydrolysates, as the sweeteners; polysaccharides, such locust bean gum, guar gum, carboxymethyl cellulose, and/or carrageenan, as the stabilizers; monoglycerides and diglycerides and polysorbate 80, as the emulsifiers; and milk or water, as the main sources of water in the formulation to balance the components (Marshall *et al.*, 2003). Usually, one mix is used for the production of a variety of flavors.

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.



**Figure 12.1.** Flow diagram for the production of ice cream.

The manufacturing process for most of these products is similar and involves the following steps (Figure 12.1): preparation of a liquid mix by blending of ingredients, pasteurization (65°C for 30 min or 80°C for 25 s), homogenization, cooling to 4°C and ageing of the cold, liquid mix for 4–24 h; concomitantly whipping and freezing this mix dynamically under high shear to a soft, semi-frozen slurry with an air phase volume of 45–52% (overrun of 80–110%) at a temperature of about –5°C; incorporation of flavoring ingredients to this partially frozen mix; packaging the product; and further quiescent freezing (hardening) of the product in blast air to –30°C (Marshall *et al.*, 2003). Homogenization is responsible for the formation of the fat emulsion by forcing the hot mix through a small orifice under a pressure of 14–18 MPa, perhaps with a second stage of 3–4 MPa. Ageing allows for hydration of milk proteins and stabilizers (some increase in viscosity occurs during the ageing period), crystallization of the fat globules, and membrane rearrangement due to competitive displacement of adsorbed proteins by low molecular mass surfactants. The concomitant aeration and freezing process involves numerous physical changes, including

the action of proteins and surfactants in forming and stabilizing the foam phase, partial coalescence of the fat emulsion, causing both adsorption of fat at the air interface and the formation of fat globule clusters that stabilize the lamellae between air bubbles, and freeze concentration of the premix by the removal of water from solution in the form of ice.

## 12.2. Sources of Fat in Ice Cream

The fat component of frozen dairy dessert mixes increases the richness of flavor, is a good carrier and synergist for added flavor compounds, produces a characteristic smooth texture by lubricating the palate, helps to give structure through the process of partial coalescence and foam stabilization and aids in producing desirable melting properties (Marshall *et al.*, 2003). The fat content is an indicator of the perceived quality and/or value of ice cream. Ice cream must have a minimum fat content of 10% in many legal jurisdictions. Premium ice creams generally have a fat content of 14–18%. It has also become desirable, however, to produce light ice creams, <10% fat (carrying such descriptors as reduced-fat, light, low-fat, and non-fat or fat-free, as appropriate for the legal jurisdiction), with the same perceived quality, if both structure and flavor considerations can be satisfied by other means.

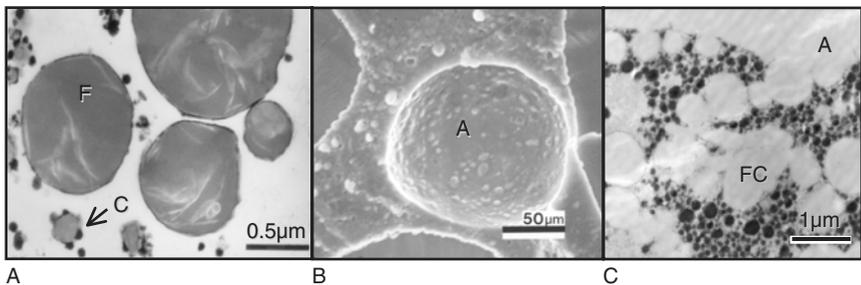
The use of milk-fat as a fat source for ice cream formulations is widespread in North America, Australia, New Zealand and much of Europe. The triglycerides in milk fat have a wide melting range (+40° to -40°C). The crystallization pattern of milk fat is also very complex, due in part to the large variation in fatty acids and the large numbers of different triglycerides present. Consequently, there is always a combination of liquid and crystalline fat at refrigeration and sub-zero temperatures, which is critical for structure formation, as will be discussed subsequently. The volatile, short-chain fatty acids also contribute to the unique flavor of milk fat. The best source of milk fat in ice cream for high quality flavor is fresh cream. Other sources of milk fat include sweet (unsalted) butter, anhydrous milk fat (butter oil), frozen cream, or condensed milk blends. Whey creams have also been used but may lead to flavor or texture problems.

Vegetable fats are used extensively as fat sources in ice cream in the United Kingdom, parts of Europe, the Far East and Latin America but only to a very limited extent in North America. Five factors of great interest in the selection of fat sources are: (1) the crystal structure of the fat, (2) the rate at which the fat crystallizes during dynamic temperature conditions, (3) the temperature-dependence of the melting profile of the fat, especially at chilled and freezer temperatures, (4) the content of high melting-point triglycerides (which can cause a waxy, greasy mouthfeel) and (5) the flavor and purity of

the oil. It is important that the fat droplets contain an intermediate ratio of liquid: solid fat at the time of freezing. It is difficult to quantify this ratio as it is dependent on a number of compositional and manufacturing factors; however, 50–67% crystalline fat at 4–5°C is a good working rule (Berger, 1997). Palm kernel oil, coconut oil, palm oil, and fractions thereof, plus their hydrogenated counterparts, or blends of oils are often used in ice cream manufacture, selected to take into account physical characteristics, flavor, availability, stability during storage and cost.

### 12.3. Contribution of Fat to the Structure of Ice Cream

The texture of ice cream is one of its most important quality attributes. Texture is the sensory manifestation of structure; thus, establishment and maintenance of optimal ice cream structure are critical to maximal textural quality. The colloidal structure of ice cream begins with the mix as a simple emulsion, with a discrete phase of partially crystalline fat globules surrounded by an interfacial layer comprised of proteins and surfactants (Figure 12.2A). The continuous serum phase consists of unadsorbed casein micelles in suspension in a solution of sugars, unadsorbed whey proteins, salts and high molecular weight polysaccharides. During the dynamic freezing stage of manufacture, the mix emulsion is foamed, creating a dispersed phase of air bubbles (Figure 12.2B), and is partially frozen, forming another dispersed phase of ice crystals. Air bubbles and ice crystals usually range in size from 20–50  $\mu\text{m}$  and are surrounded by a temperature-dependent unfrozen continuous matrix of sugars, proteins, salts, polysaccharides and water (Goff, 1997, 2002). In addition, the partially crystalline fat phase in



**Figure 12.2.** The structure of ice cream mix and ice cream. (A). Fat globules (F) in mix with crystalline fat within the globule and adsorbed casein micelles (C), as viewed by thin section transmission electron microscopy. (B). Close-up of an air bubble (A) with adsorbed fat, as viewed by low temperature scanning electron microscopy. (C). Air bubble (A) with adsorbed fat cluster (FC) that extends into the unfrozen phase, as viewed by thin section transmission electron microscopy with freeze substitution and low temperature embedding.

the mix at refrigeration temperatures undergoes partial coalescence during the concomitant whipping and freezing process, resulting in a network of agglomerated fat (Boode and Walstra, 1993; Boode *et al.*, 1993), which adsorbs to the air bubbles and extends into the unfrozen phase, producing a fat network structure throughout the product (Figure 12.2C).

The development of structure and texture in ice cream is sequential, basically following the manufacturing steps. To describe the role of fat in the structure thoroughly, it is necessary to begin with the formation of the emulsion at the time of homogenization and the role of the ingredients present at the time of homogenization, with particular reference to the fat, proteins and emulsifiers. After preheating or pasteurization, the mix is at a temperature sufficient to have melted all the fat present, and the fat is passed through one or two homogenizing valves. The creation of a large population of small, discrete droplets is a prerequisite for the development of structure during dynamic freezing, utilizing these droplets. Thus, homogenization conditions can have a large impact on ice cream structure (Koxholt *et al.*, 2001; Ruger *et al.*, 2002; Hayes *et al.*, 2003; Olsen *et al.*, 2003). Immediately following homogenization, the newly formed fat globules are practically devoid of membranous material and readily adsorb amphiphilic molecules from solution, including casein micelles, non-micellar  $\beta$ -casein, whey proteins, phospholipids, lipoprotein molecules, components of the original milk fat globule membrane and any added surfactants. All these species compete for space at the fat surface. By controlling the amphiphilic material present at the time of homogenization, it may be possible to predetermine the adsorbing substances and thus create a membrane with more favorable functional attributes, utilizing natural proteins rather than relying on the low molecular mass surfactants (Segall and Goff, 1999, 2002a,b). The membrane formed during homogenization continues to develop during the ageing step and rearrangement occurs until the lowest possible energy state is reached (Barfod *et al.*, 1991). The transit time through a homogenization valve is in the order of  $10^{-5} - 10^{-6}$  s (Pandolfe, 1982). Protein adsorption or unfolding at the newly formed interface may take minutes or even hours to complete. It is clear, therefore, that the membrane formed immediately upon homogenization is a function of the microenvironment at the time of its creation and that the recombined membrane of the fat globule in the aged mix is not fully developed until well into the ageing process (Gelin *et al.*, 1994, 1996a,b).

Low molecular mass surfactants are not needed in an ice cream mix to stabilize the fat emulsion, due to an excess of protein and other amphiphilic molecules in solution. If a mix is homogenized without added surfactants, both the whey proteins and the caseins will form this new fat globule membrane, with the caseins contributing most of the adsorbed protein

(Gelin *et al.*, 1994, 1996a,b). However, if added surfactants, such as mono-glycerides or sorbitan esters, are present, they have the ability to reduce the interfacial tension between the fat and the water phases to a lower value than proteins. Thus, they are preferentially adsorbed to the surface of the fat and the mixed membrane of surfactant and protein gives rise to the appropriate membrane for subsequent partial coalescence of the fat globules (Goff *et al.*, 1987; Goff and Jordan, 1989; Barfod *et al.*, 1991; Pelan *et al.*, 1997; Davies *et al.*, 2000, 2001; Sourdet *et al.*, 2002, 2003). As the interfacial tension is lowered and proteins are eliminated from the surface of the fat, reducing the surface excess (quantity of adsorbed material,  $\text{mg}/\text{m}^2$ ), the actual membrane becomes weaker to subsequent destabilization due to this reduction of steric stabilization, although the emulsion is thermodynamically favored due to the lowering of the interfacial tension and the net free energy of the system. Fat globules with reduced steric stabilization also adsorb at air interfaces, enhancing foam stability (Goff *et al.*, 1999; Zhang and Goff, 2004).

Crystallization of fat also occurs during ageing, creating a highly intricate structure of needle-like crystals within the globule. Triglycerides with a high melting point crystallize first, and continue to be surrounded by liquid oil of those triglycerides with lower melting points. Crystallization of emulsified milk fat at refrigeration temperature reaches equilibrium within 1.5 h (Adleman and Hartel, 2002; Relkin *et al.*, 2003). A partially crystalline fat droplet is necessary for optimal fat structure formation to occur during freezing (Davies *et al.*, 2000, 2001). Stability of a paraffin oil-in-water emulsion was reduced by six orders of magnitude when crystals were present in the dispersed phase compared to liquid oil droplets (van Boekel and Walstra, 1981). This has been attributed to the protrusion of crystals into the aqueous phase, which cause a surface distortion of the globule. These protrusions can pierce the film between two globules upon close approach. As the crystals are preferentially wetted by the lipid phase, clumping is inevitable.

The next stage of structure development occurs during the concomitant whipping and freezing step. Air is incorporated either through a lengthy whipping process (batch freezers) or as it is drawn into the mix by vacuum (older continuous freezers) or is injected under pressure (modern continuous freezers). The air bubbles are formed through a combination of comminution and interfacial adsorption (Chang and Hartel, 2002a). If the fat globules are sufficiently unstable to shear, as a result of reduction of membrane surface excess and steric stabilization due to added small-molecule surfactants, the aeration and ice crystallization processes cause the emulsion to undergo partial coalescence or fat destabilization, during which clusters of fat globules form and build an internal fat structure or network in the frozen product. Bolliger *et al.* (2000a) showed a direct relationship between protein

content ( $\text{mg}/\text{m}^2$ ), resulting from displacement by emulsifiers, and partial coalescence. The incorporation of air alone, or shearing action alone, independent of freezing, are not sufficient to cause the high degree of fat destabilization that occurs when ice crystallization and air incorporation occur simultaneously (Kokubo *et al.*, 1996, 1998; Chang and Hartel, 2002b).

Fat destabilization results in the following beneficial properties: dryness (shape retention) upon extrusion during the manufacturing stages (which facilitates packaging and novelty molding, for example), a smooth, creamy texture in the frozen dessert, and resistance to melt-down or good stand-up properties (necessary for soft serve operations) (Bolliger *et al.*, 2000a; Goff and Spagnuolo, 2001). The clusters of fat globules formed during the process of partial coalescence are responsible for adsorbing to, and stabilizing, the air cells (Turan *et al.*, 1999; Goff *et al.*, 1999; Barfod, 2001; Zhang and Goff, 2004) and creating a semi-continuous network or matrix of fat throughout the product that crosses the lamellae between the air cells (Koxholt *et al.*, 2001; Muse and Hartel, 2004). Hence, a finer distribution of air bubbles, resulting in thinner lamellae, also helps to produce optimal shape retention during extrusion and melting (Bolliger *et al.*, 2000b). Optimal formation of fat structure and air bubble size may also help to slow down ice recrystallization (Barfod, 2001). If an ice cream mix is subjected to excessive shearing action or contains too much emulsifier, the formation of objectionable butter particles can occur as the emulsion is churned beyond the optimum level.

#### **12.4. Contribution of Fat to Ice Cream Texture and Flavor**

Fat contributes greatly to the flavor and texture of ice cream. Several studies have discussed flavor and textural aspects of various fat sources (Abd El-Rahman *et al.*, 1997), non-fat sources to provide fat-like properties ("fat replacers"; Ohmes *et al.* 1998; Adapa *et al.*, 2000; Aime *et al.*, 2001) and the effect of fat on sensory properties (Guinard *et al.*, 1997; Roland *et al.*, 1999; Prindiville *et al.*, 1999) and flavor perception (Li *et al.*, 1997; Hyvonen *et al.*, 2003) of ice cream.

In addition to positive aspects, numerous flavor and textural defects may be associated with the fat phase of ice cream. Such flavor defects are usually related to either autoxidation of the fat, resulting in oxidized flavors (cardboardy, painty, metallic) or, especially in the case of milk-fat, lipolysis of free fatty acids from triglycerides by the action of lipases (referred to as hydrolytic rancidity). A significant content of free butyric acid gives rise to very undesirable rancid flavors. These defects tend to be present in the raw ingredients used in ice cream manufacture, rather than promoted by the ice cream manufacturing process itself. However, processing

problems can also occur during the preparation of ice cream mix (e.g., rapid agitation/foaming of raw milk or cream), that can give rise to these fat-related flavor defects (Marshall *et al.*, 2003).

The fat phase can also account for textural defects associated with the fat content (too high or too low) or degree of partial coalescence. Fat contributes smoothness to the finished product. Low-fat mixes must therefore compensate for this lack of inherent smoothness by altering the ratio of other components, particularly the protein, polysaccharide stabilizer, and emulsifier components. On the other hand, mixes high in fat, such as the premium products, typically have a heavier, dense texture, related to both the high fat and the lower-than-normal air content. Partial coalescence of the fat emulsion modifies the textural perception, giving ice cream a more creamy texture, in addition to its role in structure. If too much destabilization has occurred, the ice cream will taste greasy and a defect known as “does not melt” may occur (Marshall *et al.*, 2003). This results from a network of fat that gives sufficient structure to the product to hold its shape without collapse in the absence of the ice phase, after warming to a temperature sufficient to melt the ice.

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# Significance of Milk Fat in Milk Powder

N.Y. Farkye

## 13.1. Introduction

The production of milk powder involves dehydration *via* evaporation and spray drying, followed by packaging and storage. The two principal types of milk powders produced directly from milk or skim milk are whole (full-cream) milk powder (WMP) and skimmed milk powder (SMP; or non-fat dry milk, NDM). There are three types of SMP—low-heat, medium-heat, and high-heat—that are classified based on the level of undenatured whey proteins (whey protein nitrogen, WPN index), which is a direct result of the level of heat treatment given to milk/concentrate during powder manufacture. The WPN index for low-heat, medium-heat and high-heat powders is, respectively,  $\geq 6.0$ , 1.51–6.0 and  $\leq 1.5$  mg WPN/g (ADPI, 2002). Other dried milk products containing fat are buttermilk powder (BMP), fat-filled milk powder, cream powder, retentate powder, and cheese powder. The majority of milk powder produced worldwide is in countries with a temperate climate and a relatively large dairy industry. The powder is exported mostly to tropical and developing countries in which the dairy industry is not well developed and facilities for cooling and handling fresh milk are inadequate. In milk powder-producing countries, some of the milk powder is used in a variety of dairy and food applications (e.g., ice cream, cheese, infant formulae, evaporated milk, sweetened condensed milk). Milk powder is also used as an ingredient in bakery products, processed meats, soups, etc. The function of milk powder in these products is influenced by the components of the powder, primarily protein (casein or whey proteins), fat and lactose. The significance of milk fat in milk powder is the subject of this review, and it is focused on WMP.

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

Figure 13.1 is a flow diagram for the traditional processes for the manufacture of WMP, SMP, and BMP. An alternate process for the manufacture of WMP that involves blending highly heated cream and low heat-treated skim milk concentrate prior to drying, was described by Hols and Van Mil (1991). The recommended standards for WMP, SMP and BMP, as well as average composition of the powders, are shown in Table 13.1.

## 13.2. Overview of Milk Powder Manufacture

Each step in the processing and packaging of dry milk products can influence the properties of the finished product. The total heat treatment (i.e., time  $\times$  temperature) for each step during the manufacture of milk powder is important in determining its properties. The method of heating and the total heat exposure influence protein denaturation, oxidation of the fat and the subsequent shelf-life of the powder.

Following preheat treatment (pasteurization and homogenization), the milk is concentrated to about 45–55% dry matter by vacuum evaporation and the concentrate atomized and dried by spray or drum (roller) drying methods. Currently, the most commonly used method for drying milk products is spray drying. However, any method used for drying must remove water from the milk concentrate as fast and at as low a temperature as possible to minimize heat damage to the milk solids. The atomizing device influences the size, shape, and density of the particles. During spray drying, the milk and concentrate are exposed to varying degrees of heat, depending on the dryer type, time of exposure, and operating conditions. The drying conditions influence the physical and chemical properties of milk components.

The physico-chemical properties of milk powder are determined by the composition of the raw milk (Buma and Henstra, 1971; Aguilar and Ziegler, 1994a,b) and the manufacturing conditions, (i.e., method of concentration, feed temperature, pump pressure, the inlet and outlet temperature of the dryer) (Verhey, 1972; de Vilder *et al.*, 1976; Fitzpatrick, O'Callaghan, 1996), packaging, and storage conditions. Some properties such as flavor, appearance, free fat content (for WMP), instantization, solubility, heat stability, flowability, dispersibility, wettability, and bulk density have practical implications. The reconstitutability of milk powder is related to the particle size.

Milk powder particles  $< 100 \mu\text{m}$  in size are often difficult to wet and tend to become lumpy when dispersed in water (Schubert, 1987). Hence, milk powder is agglomerated during manufacture to make the powder instantly wettable in warm ( $> 45^\circ\text{C}$ ) water. Agglomeration is the process of forming porous clusters of powder particles to increase the volume of occluded air, thereby increasing its dispersibility and making the powder

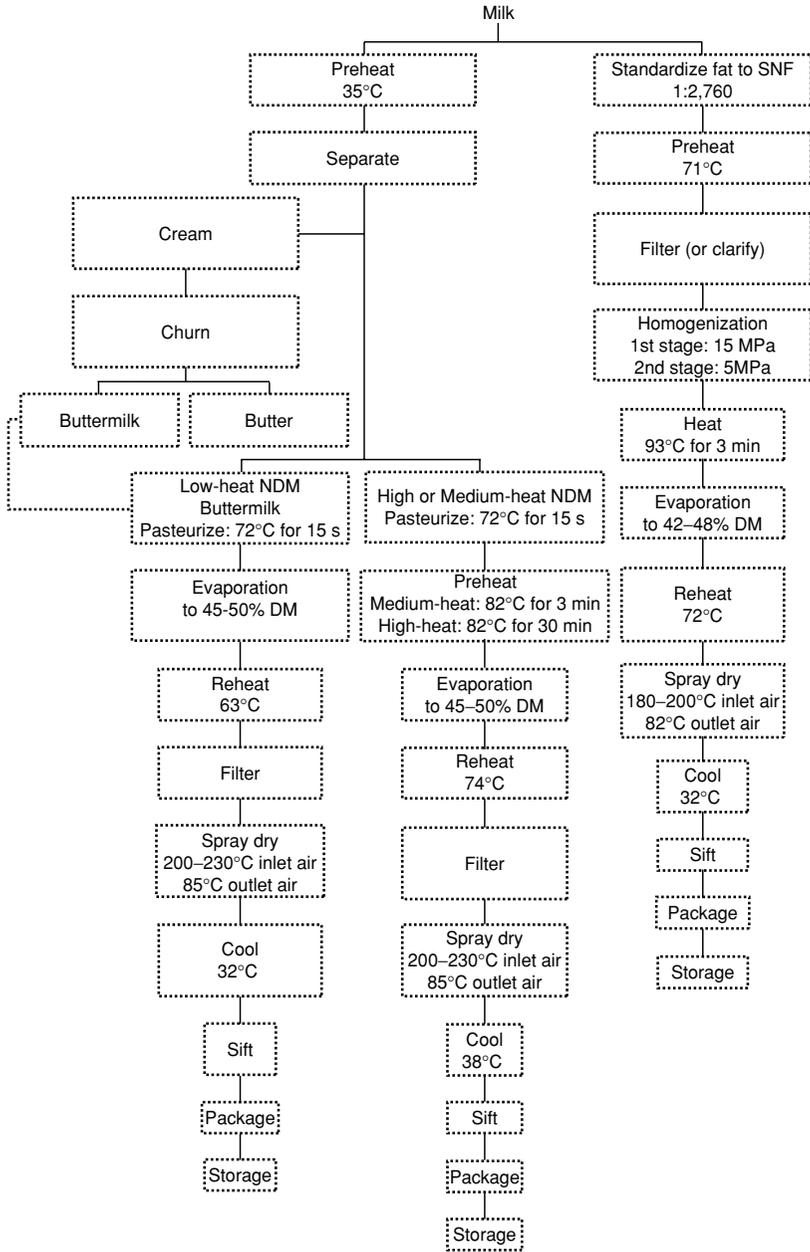


Figure 13.1. Flow diagram for the production of whole milk, skim milk and buttermilk powder.

**Table 13.1.** Recommended (ADPI, 2002) and actual Mean Composition of Skim Milk, Buttermilk and Whole Milk Powders (USDA, 1999)

Component	Whole milk powder	Skim milk powder	Buttermilk powder
<b>Recommended</b>			
Protein, %	26.0	36.0	34.0
Fat, %	Min. 26–40 (max.)	Max. 1.25	Max. 4.5
Moisture, %	Max. 4.5	Max. 4.0	Max. 4.0
Lactose, %	37–40	49–54	47–51
Ash, %	5.3–6.3	7.4–8.4	7.0–8.0
Titratable acidity, % lactic acid	0.15	0.15	0.10–0.18
Solubility index, ml <sup>a</sup>	1.0 <sup>a</sup>	1.25 <sup>a,b</sup>	1.25 <sup>b</sup>
<b>Actual</b>			
Protein, %	26.3	36.2	34.3
Carbohydrate (lactose), %	38.4	52.0	49.0
Ash, %	6.1	7.9	8.0
Dry matter, %	97.5	96.8	97.0
Moisture, %	2.5	3.2	3.0
Fat, %	26.7	0.8	5.8
Fatty acids, total saturated, %	16.5	0.5	3.6
4:0	0.87	0.03	0.19
6:0	0.24	0.01	0.11
8:0	0.27	0.01	0.07
10:0	0.60	0.02	0.15
12:0	0.61	0.01	0.16
14:0	2.82	0.08	0.58
16:0	7.52	0.24	1.52
18:0	2.85	0.09	0.70
Fatty acids, total monounsaturated	7.92	0.20	1.67
16:1	1.20	0.02	0.13
18:1	6.19	0.17	1.45
Fatty acids, total polyunsaturated	0.67	0.03	0.22
18:2	0.46	0.02	0.13
18:3	0.20	0.01	0.08

<sup>a</sup> Higher (2.0 ml) for high-heat powders and powder manufactured by roller drying.

<sup>b</sup> Higher (15 ml) for powder manufactured by roller drying.

easy to dissolve. However, to improve the wettability of WMP: to give it cold-water instant properties, it is treated with a mixture of lecithin and butter oil. Powdered lecithin is dissolved in butter oil to give a 25–50% solution. The warm (65–70°C) lecithin solution (as a wetting agent) is sprayed onto the powder particles to give a final lecithin content of 0.2% in the powder (Kyle, 1993; Pisecky, 1997).

### 13.3. Significance of Milk Fat during Powder Manufacture

Milk fat is undoubtedly the most important source of flavor and off-flavor compounds associated with milk powders. It plays a role in the behavior of powder during reconstitution and also contributes to potential hazards during powder drying. Auto-oxidation of fat during the drying of milk can cause self-ignition of dry milk deposits in the dryer and is the most likely cause of fire and explosion in milk powder factories (Knipschildt, 1986). Fat-filled powders oxidize readily at the exit air temperature of 80–90°C; the rate of oxidation at a given temperature increases with the degree of unsaturation. Ignition depends on the thermal conductivity of the powder and the rate at which milk powder absorbs and dissipates heat (Walker and Jackson, 1978). The thermal conductivity of WMP increases with temperature and bulk density. For WMP with bulk density of 0.512 g/cm<sup>3</sup>, thermal conductivity increases from 0.036 W/mK at 11.8°C to 0.077 W/mK at 43.2°C, while at a bulk density of 0.605 g/cm<sup>3</sup>, the thermal conductivity increases from 0.058 W/mK at 16.6°C to 0.093 W/mK at 42.8°C (MacCarthy, 1985).

WMP is traditionally sold for use in various food applications and end-uses that sometimes require powder with certain properties. For example, when used for chocolate manufacture, WMP must have a high free fat content, large particle size and low vacuole volume (Keogh *et al.*, 2003). Contrarily, for other products, WMP with a high level of free fat is undesirable due to rapid development of oxidized flavors, tallowness, poor flowability and the formation of a fat layer on the surface of reconstituted solutions leading to “dead” powders (Pisecky, 1997). Traditionally, WMP for chocolate manufacture is produced by drum (or roller) drying because of the resultant high (~85–95% as a percentage of total fat) free fat content compared to 1–1.5% for powder manufactured by spray drying (Pisecky, 1997; Keogh *et al.*, 2003). The large surface area of milk powder particle results in a high free fat content, therefore, the concentrate is homogenized prior to drying to reduce the amount of free fat.

Fat in dried milk is present in a finely emulsified state or in a coalesced de-emulsified state (Buma, 1971). Damage or removal of the fat globule membrane causes the fat globules to flow together as “pools” (Buma, 1971). Free fat is defined as the fraction of fat that is not protected by protein film but is present as “fat pools” or “patches” instead of globules on the surface of whole milk powders (Pisecky, 1997). It is the fraction of milk fat that is on the surface of the powder particle, or in vacuoles, pores, or cracks, and is extractable under defined conditions, (i.e., solvent type, extraction time and temperature) (Buma, 1971). Pisecky (1997) summarized the work of Buma (1971), which describes four types of extractable fat as:

- Surface fat—which is present as pools or patches on the surface of the particles
- Outer layer fat—formed by fat globules that almost touch the particle surface and are easily reached by solvents
- Capillary fat—fat globules that touch the surface of micropores and cracks and, therefore, are easily reached by solvents
- Dissolution fat—fat globules that touch any holes remaining after dissolving the outer layer.

The amount of extractable fat depends on the moisture content of the powder. When the moisture content of milk powder is increased from 2 to 5%, the amount of free fat decreases, but when the moisture content is above 6–7%, the free fat content increases (Buma, 1971). In regular WMP, the continuous phase of milk powder particles is amorphous lactose and other serum constituents, which form a tight membrane that protects the fat globule from extraction. Therefore, to manufacture spray-dried WMP with a high free fat content, most of the lactose is crystallized as  $\alpha$ -lactose monohydrate (Pisecky, 1997). Spray-dried milk powder particles are generally spherical (ranging from 10 to 250  $\mu\text{m}$  in diameter) with mostly a smooth surface but with some folds and wrinkles, and air bubbles (Saito, 1985; Aguilar and Ziegler, 1994a,b). A rougher particle surface and increased porosity results when powders are manufactured with a high temperature gradient between the atomized milk mist and the hot drying air. This contributes to better agglomeration properties of the powder.

The amount of free fat in powder is inversely related to particle size (hence large surface area), directly to the number of vacuoles and to the drying temperature (a high temperature causes more cracks) (Buma, 1971). Other methods for producing spray-dried WMP with high free fat include spray-drying part of the fat onto a partly skimmed spray-dried powder (Verhey, 1986) or blending or co-drying high-free fat milk powder containing a high fat content with SMP to obtain WMP containing 26% fat (Twomey *et al.*, 2000). Also, spray dried WMP with high free fat can be produced by ultrafiltration and fat standardization of milk before evaporation and drying (Keogh *et al.*, 2003). The free fat content of milk powder is influenced by a number of factors (Pisecky, 1997):

- Total fat content—free fat content increases with fat content above 26%.
- Type of fat—use of vegetable fat with a low melting point.
- Product composition—a high lactose content gives low free fat; a high protein content gives high free fat.
- Physical state of lactose—amorphous lactose protects free fat from being extracted while crystalline lactose promotes extraction.

- Drying conditions—gentle drying results in powder particles with a smooth surface and hence a lower level of free fat. High-temperature drying results in cracks and micropores.
- Powder handling—gentle treatment reduces the level of free fat; hence, avoid pneumatic transport, use a type of dryer with a low cyclone fraction, operate with a low pressure drop over cyclones, allow adequate powder cooling in fluid bed, and avoid high powder moisture from first drying stage.
- Standardization—standardization with buttermilk should be avoided.
- Feed concentration—up to a level which insures good solubility index.
- Lecithin—lecithin should not be used as emulsifier for fat-filled powders.
- Extent of drying—over-drying to too low a moisture content should be avoided.
- Homogenization—two-stage homogenization at medium pressures should be used.
- Addition of crystalline lactose—when crystallized lactose is added, it must be dissolved completely.

### 13.4. Significance of Milk Fat for the Flavor of Milk Powder

The most significant deteriorative changes that occur in dry milk products that result in sensory, functional, and visual defects, are oxidation of the milk lipids and the reducing sugar-protein browning, or Maillard, reaction. The oxidative stability of milk-fat in milk powder is influenced by the heat treatment of the milk, storage temperature, water activity (Stapelfeldt *et al.*, 1997) and packaging (Tuohy *et al.*, 1981; Mrithyunjaya and Bhanunurthi, 1987).

#### 13.4.1. Effect of Pre-heat Treatment on Oxidative Stability

Low-heat powders are more susceptible to severe oxidative changes during storage than medium-heat and high-heat powders (Stapelfeldt *et al.*, 1997). Pre-heating of milk generates free SH groups, which act as antioxidants. Kirchmeier *et al.* (1984) reported that the formation of free SH groups starts at 72°C and reaches a maximum at 95°C. Rotkiewicz *et al.* (1979) reported that the amount of free SH groups in reconstituted milk powder manufactured from milk that was pre-heated to 65, 80 or 95°C for 30 s was 10.3, 30.5 and 54.9  $\mu\text{mol/l}$ , respectively. After 5 months of storage at 30°C, the amount of free SH groups were 6.8, 15.3 and 42.3, respectively (Rotkiewicz *et al.*, 1979). Kumar and Murthy (1995) also reported a net

decrease of 1.62–1.77  $\mu\text{mol/g}$  in buffalo WMP after 12 months storage at ambient temperature (22–38°C). Baldwin *et al.* (1991) observed increased stability during storage and improved flavor of milk powder when a moderate pre-heat treatment at >95°C for about 20 s was applied to the milk and the powder and stored in air under atmospheric conditions. Also, WMP manufactured from milk that was preheated at 110°C for 30 s had a lower peroxide value than that made from milk pre-heated at 95°C for 2 min (van Mil and Jans, 1991). A combination of pre-heating, avoidance of copper contamination, and gas flushing to the lowest attainable oxygen content in the package minimize oxidative deterioration.

Oxidized flavor and aroma in WMP can be prevented or retarded by increasing the level of preheat treatment during powder manufacture. However, excessive pre-heating may itself induce off-flavors and may not be always beneficial to the overall product quality. Touhy and Kelly (1989) found that the oxidative stability of WMP is related to the conditions under which natural gas is combusted. Direct heating of the drying air by direct firing of liquid petroleum gas and natural gas for the manufacture of WMP resulted in a thiobarbituric acid (TBA) value of 0.037–0.054 compared to 0.016–0.018 for powders produced by indirect heating using a conventional gas burner (Tuohy and Kelly, 1989).

#### **13.4.2. Influence of Moisture Content and Water Activity on the Oxidation of Fat in Milk Powder**

The maximum shelf-life of bulk packaged WMP containing ~3% moisture is about 6 months at 30°C (Kjærgaard Jensen, 1988). The oxidation of WMP, as measured by peroxide value, is dependent on the moisture content of the powder. van Mil and Jans (1991) reported that under similar storage conditions, the peroxide value of WMP increases more rapidly for powder containing 3% moisture than in powder containing 2.4% moisture. The water activity ( $a_w$ ) range for WMP is usually 0.13–0.20, with a typical value from 0.16 to 0.18 (Wewala, 1990). Stapelfeldt *et al.* (1997) found that the quality of WMP is maintained best at  $a_w$  between 0.11 and 0.23, whereas the quality of the powder decreases when stored at  $a_w$  of 0.31 at 45°C. However, the critical  $a_w$  for improved oxidative stability of WMP stored at 40°C for one year is 0.21–0.24 at a moisture level of 3.4% (Wewala, 1990).

#### **13.4.3. Effect of Oxygen Content and Packaging the Oxidation of Fat in Milk Powder**

Packaging materials play a vital role in controlling and/or minimizing any adverse changes that may occur during the storage of milk powder. It is

important to use packaging material that is strong enough to resist mechanical and physical damage to the product during handling, distribution, and storage. Packaging materials must be relatively impervious to moisture vapor, must not transmit volatile by-products, must protect the product from contamination and microorganisms and must be easy to seal.

Different types of packaging and packaging materials have been investigated for milk powder (Mrithyunjaya and Bhanumurthi, 1987; Ipsen and Hansen, 1988; Lim *et al.*, 1994). A general specification for a 25 kg package is a Kraft paper multiwall bag (not less than  $420 \text{ g m}^{-2}$  with an outer ply incorporating a moisture barrier laminate) and an inner low-density polyethylene liner (Nichols, 1979). The outer ply consists of two layers of Kraft paper (either crepe or natural) and inner ply of the bag is natural Kraft paper ( $85 \text{ g m}^{-2}$ ). The inner polyethylene ply should be  $76 \mu\text{m}$  thick.

The properties of milk powder such as solubility index, free fat content, hydroxymethylfurfural (HMF) value and TBA value are influenced by the type of package used. The sensitivity of WMP to oxygen has a major influence on its storage stability. Coulter (1948) reported that drying of milk powder in an atmosphere of inert gas has no effect on its shelf-life but that the level of oxygen in the package influences the shelf-life of the powder. At an  $\text{O}_2$  level of 1%, WMP stores well both at room temperature or higher (Lea *et al.*, 1943). Peters (1974), who gas-flushed WMP in a pouch with 8%  $\text{H}_2$  and 92%  $\text{N}_2$ , found that the  $\text{O}_2$  content in the pouch was almost zero. Milk powder packed in 92%  $\text{N}_2$  and 8%  $\text{H}_2$  in the presence of a palladium catalyst contained low levels of volatile compounds during storage (Min *et al.*, 1989).

van Mil and Jans (1991) studied the properties of WMP that was gas-packed ( $\text{CO}_2/\text{N}_2$ ) at three levels of  $\text{O}_2$  (21, 5–7 and  $<3\%$ ). They reported that the powder packed at an  $\text{O}_2$  content of 5–7% or  $<3\%$  maintained its flavor throughout storage. However, at a high (21%)  $\text{O}_2$  content, the peroxide value increased and flavor score decreased with time and temperature of storage. The formation of Strecker aldehydes in cream powders is  $\text{O}_2$ -dependent but the formation of 2-alkanones is not (Anderson and Lingnert, 1997). van Mil and Jans (1991) also reported that WMP manufactured in spring in the Netherlands is more susceptible to autoxidation than that manufactured during other seasons. Tuohy *et al.* (1981) reported that WMP packed in tin cans was stable for 12 months at  $12^\circ\text{C}$  but the shelf-life was 24 weeks at  $37^\circ\text{C}$ .

To minimize and control oxidation of milk-fat in milk powder, Van Mil and Jans (1991) suggests:

- Using good quality milk with low total bacterial and coliform counts
- Raw milk with a low copper content ( $<100 \mu\text{g}/\text{kg}$ )

- Pre-heating milk to 90–95°C for 1 min to maximize—SH groups and an acceptable level of cooked off-flavor
- Reducing the O<sub>2</sub> content in the package

Oxygen scavengers can be used to reduce the O<sub>2</sub> content in packaged milk powder but this approach may not be cost-effective (Zimmerman *et al.*, 1974). Another approach to reducing the O<sub>2</sub> content in the package involves using H<sub>2</sub> in the presence of a palladium or platinum catalyst. Although the use of several antioxidants is permitted in the manufacture of edible oils, fats and butter, their use in milk powder is prohibited.

Experimentally, anti-oxidants such as gallic acid, ethyl gallate, oat flour (Findlay and Smith, 1945), sorbitan monostearate, glycerol monostearate, polyoxyethylene sorbitan monostearate (Hibbs and Ashworth, 1951), ascorbyl palmitate, dodecyl gallate (Abbot, 1971), high-oryzanol rice bran oil – containing tocopherols and tocotrienols (Nanua *et al.*, 2000), nordihydroguaiaretic acid, butylated hydroxyanisole, butylated hydroxy toluene and citric acid (Helal *et al.*, 1976) have been used to enhance the oxidative stability of milk powders.

#### 13.4.4. Lipid Oxidation Products

Oxidation of fat in WMP is measured by an increase in the peroxide value, which is normally ~0.17 – 0.21 meq/kg fat in fresh WMP (Newstead and Headifen, 1981; Hols and Van Mil, 1991) or by the TBA assay for secondary oxidation products, which causes off-flavors (Ulberth and Roubicek, 1995). There is a strong positive correlation ( $r = 0.931$ ) between peroxide value and the hexanal content of WMP (Ulberth and Roubicek, 1995).

The oxidation of unsaturated fatty acids in WMP results in the formation of saturated aldehydes (Boon *et al.*, 1976), whereas the oxidation of polar lipids generates unsaturated aldehydes and ketones (Kinsella, 1969). The saturated aldehydes are responsible for “stale,” “tallowy,” “cardboard” and “painty” flavors whereas unsaturated aldehydes with a very low threshold also contribute to oxidized flavors (Keen *et al.*, 1976; Hall and Anderson, 1985). The concentration of total monocarbonyls was 4.35 and 11.45  $\mu\text{mol}/100\text{ g}$  in air-packed WMP analyzed 1 h after drying and after 16 months storage at 37°C, respectively (Boon *et al.*, 1976). The concentration of monocarbonyls in WMP that was nitrogen-packed in sealed cans and stored for 16 months at 37°C was 6.69  $\mu\text{mol}/100\text{ g}$  (Boon *et al.*, 1976). Most of the oxidative carbonyls belong to four classes, namely, n-alkanals, 2-alkanals, alk-2,4-dienals, and 2-alkanones (Kinsella, 1969). Of these, 2-alkanones and n-alkanals represent over 85% of the total monocarbonyls in WMP (Boon *et al.*, 1976). Freshly

produced WMP contains  $< 10 \mu\text{g}/\text{kg}$  hexanal whereas samples stored for 130 days at  $30^\circ\text{C}$  contained  $106 \mu\text{g}/\text{kg}$  (Ulberth and Roubicek, 1995). There is a positive correlation ( $r = 0.89$ ) between the concentration of hexanal that is a secondary oxidation product of linoleic acid, and the presence of cardboard-like off flavors (Hall and Lingnert, 1986). Unsaturated aldehydes at low but organoleptically-significant concentrations also occur in oxidized milk powders, with 2,4-undecadienal being the most abundant unsaturated aldehyde present. However, other compounds such as 2-pentenal, 2-heptenal, 2-nonenal, 2,4-heptadienal, and 2,4-nonadienal were present and play a role in oxidized flavor (Kinsella, 1969; Boon *et al.*, 1976).

Shiratsuchi *et al.* (1994a) identified over 187 flavor compounds in SMP: 48 hydrocarbons, 18 aldehydes, 20 ketones, 21 alcohols, 29 fatty acids, 8 esters, 2 furans, 7 phenolic compounds, 10 lactones, 14 nitrogenous compounds, and 10 miscellaneous compounds, most of which originated from the breakdown of milk fat. Among the ketones identified, methyl ketones (which possibly originate from the oxidation of free fatty acids to  $\beta$ -keto acids that are subsequently decarboxylated) were most abundant. However, the level of methyl ketones in SMP is low. For example, the flavour threshold of 2-heptanone in milk is  $700 \mu\text{g}/\text{kg}$  whereas its concentration in SMP is  $8 \mu\text{g}/\text{kg}$ . The presence of lactones (e.g.,  $\delta$ -decalactone and  $\delta$ -dodecalactone) that are characterized by milky, buttery or creamy odor, may be due to the presence of 5-hydroxy acids in bovine milk-fat (Shiratsuchi *et al.*, 1994a). Free fatty acids represent the highest proportion (79%) of total volatile compounds in SMP (Shiratsuchi *et al.*, 1994a). The flavor compounds in SMP made from off-flavored skim milk were compared to those in SMP to made from normal skim milk by Shiratsuchi *et al.* (1994b) and reported that the concentrations of aldehydes, alcohols and esters were higher in SMP from off-flavored skim milk. Compounds that contribute to off-flavor in SMP include tetradecanal (sickening and aldehydic odor),  $\beta$ -ionone (hay-like odor), and benzothiazole (sulfuric and quinone-like odor), all of which originate from milk lipids.

### 13.5. Role of Fat in the Physical Properties of Milk Powder

There is little or no information in the literature on the role of milk-fat on the bulk density, scorching particles, wettability, dispersibility and flowability of milk powder. Tuohy (1989) found considerable differences between the packed bulk density of regular SMP ( $0.85 \text{ g}/\text{cm}^3$ ), WMP ( $0.68 \text{ g}/\text{cm}^3$ ) and fat-filled milk powder ( $0.47 \text{ g}/\text{cm}^3$ ). The packed bulk density of fat-filled

milk powder was about 50% of that of SMP, suggesting that fat influences the bulk density of milk powder. Tuohy (1989) also found differences in the wettability (more than 300 s for instant SMP, 31 s for instant WMP and 131 s for instant fat-filled milk powder) and dispersibility of the various types of milk powder in air and in water. Pisecky (1980) reported wettability values ranging from 8 to 22 s, dispersibility ranging from 88 to 94% and flowability of 49 to 106 s for four commercial samples of instant WMP. It is clear that the fat content, type of fat and drying process affect the physical properties of milk powder (Tuohy, 1989).

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# Significance of Milk Fat in Infant Formulae

G. Hendricks and M. Guo

## 14.1. The Nutritional Role of Lipids

Lipids play a diverse role in human nutrition and development. They constitute a major energy source, provide an almost unlimited form of energy storage, and they act as vehicles for absorption and transport of fat-soluble compounds, such as vitamins (A, D, E, and K). Phospholipids act as functional, as well as structural, components of cell membranes. Other lipids, such as essential fatty acids, are critical for normal growth and development of the central nervous system and the retina (Uauy, 1990). Normal growth and weight-gain of infants is dependent on an adequate supply of essential fatty acids, a group of naturally occurring unsaturated fatty acids with a chain length of 18, 20, or 22 carbon atoms and containing between two and six methylene-interrupted double bonds. The human fetus, like the adult, is unable to synthesize *de novo* some of these compounds (i.e., C<sub>18:2</sub> and  $\alpha$ -C<sub>18:3</sub>) and they must therefore be supplied in the diet after birth and through the maternal plasma *in utero*. These polyunsaturated fatty acids are essential because they can be incorporated into lipid membrane structures and act as precursors in the formation of prostaglandins and related compounds, as well as providing precursors of other fat-soluble hormones.

Fat is the most variable component of human milk and although the fat content of breast milk is markedly influenced by lactation, fat composition remains relatively constant. Breast milk contains primarily fatty acids containing 10 to 20 carbon atoms. Of these, oleic (C<sub>18:1</sub>), palmitic (C<sub>16:0</sub>), linoleic (C<sub>18:2</sub>,  $\omega$ -6), and  $\alpha$ -linolenic acid (C<sub>18:3</sub>,  $\omega$ -3) are most abundant in

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

mature breast milk. The latter two are generally recognized as dietary essential fatty acids because of the inability of animal tissues to introduce the necessary double bonds in the carbon chain before carbon 9.

Human and bovine milks contain about 3–5% total lipid, existing as emulsified globules, 2–4  $\mu\text{m}$  in diameter, covered with a phospholipid–protein membrane derived from the secreting cell. About 98% of the lipids are triacylglycerols, which are found in the fat globules. Phospholipids are about 0.5–1% of total lipids and sterols are 0.2–0.5%; the phospholipids are found mostly in the globule membrane (Jensen *et al.*, 1992). (See Chapters 4 and 6).

The major differences between the lipids of bovine and human milk are in fatty acid composition and triacylglycerol structure. Bovine milk contains substantial quantities of  $\text{C}_{4:0}$ – $\text{C}_{10:0}$ , about 2%  $\text{C}_{18:2}$  and almost no other long-chain polyunsaturated fatty acids. The fatty acid composition is not altered by ordinary changes in diet. Human milk contains very little  $\text{C}_{4:0}$ – $\text{C}_{10:0}$ , 10–14% (w/w of fat)  $\text{C}_{18:2}$ , and small quantities of other polyunsaturates. The triacylglycerol structure differs, with much of the *sn*-2 position in human milk lipids occupied by  $\text{C}_{16:0}$  and the *sn*-3 position of bovine milk-fat occupied by  $\text{C}_{4:0}$ – $\text{C}_{10:0}$ .

The absorption of fats from the diet involves several steps. Hydrolysis of triglycerides by infants fed human milk is the result of the combined action of bile salt-stimulated lipase, produced in the mammary gland and secreted in the milk, and lingual and gastric lipases (Tsang *et al.*, 1993). This is extremely important to the newborn infant since there is a general lack of adequate amounts of pancreatic lipase. Moreover, milk fat globules are relatively resistant to the action of pancreatic lipase prior to hydrolysis by lingual lipase, which is able to penetrate the membrane of the milk fat globule (Suskind and Lewinter-Suskind, 1993). The slower gastric emptying and lower gastric acid level of the newborn contribute to the optimal functioning of these enzymes. Some short-chain and middle-chain fatty acids and triglycerides are absorbed directly into the portal circulation across the mucosa of the stomach (Uauy, 1990). While some milk-based commercial formulae contain these short- and middle-chain fatty acids, breast milk also contains long-chain polyunsaturated fatty acids. Among these are docosahexaenoic (DHA) ( $\text{C}_{22:6}$ ,  $\omega$ -3) and eicosapentaenoic (EPA) ( $\text{C}_{20:5}$ ,  $\omega$ -3) acids, which have been shown to be important in the development of retinal and brain tissue (Uauy, 1989).

The lipids of human milk have not been investigated as extensively as those of bovine milk. This is due largely to the economic value of bovine milk and milk products and because it is consumed by large numbers of people in all age groups. More research on human milk lipids will undoubtedly be initiated because of the revival of interest in breast-feeding and the

increased interest from groups such as the Committee on Nutrition of the American Academy of Pediatrics and the new concerns about the possible effects of early infant diet on proper growth patterns and links to the future development of obesity, diabetes, and atherosclerosis. These concerns apply particularly to many of the infant formulae now available commercially.

## 14.2. Fatty Acid Profile and Fat-Soluble Vitamins of Human Milk and Infant Requirements

The lipids in mature milks (human and bovine) can be divided into four types: triacylglycerols (triglycerides), fatty acids, sterols, and phospholipids. By far the most abundant lipids in mature milk are the triacylglycerols with much smaller quantities of sterols and phospholipids, which are present primarily in the membrane of the fat globules. The sterols are mostly cholesterol and its various esters. Traces of carotenoids, retinyl esters, and squalene are also present. Freshly drawn milk contains only trace amounts of free fatty acids (FFAs), diglycerides (DG), and monoglycerides (MG). The presence of large amounts of free fatty acids in milk samples is indicative of lipolysis which does not change the total fatty acid profile but it alters the relative amount of these compounds substantially.

The structure of the triglycerides in both human and bovine milks is also an important consideration. The structure of TGs affects their rheological properties, melting point, crystallization behavior, and absorption. The lipids in both human and bovine milk contain ten major fatty acids. Therefore, if all the fatty acids were randomly distributed, there would be in the order of 1000 possible triglycerides. The total theoretical number of possibilities is even greater, in the order of 60 million different triglycerides because bovine milk contains almost 400 fatty acids and human milk contain nearly 200 different fatty acids, although the actual number of the different molecular species has not been determined for either milk. However, one of the most important properties of the structure of the triglycerides in milk is their influence on the action of lipolytic enzymes and therefore absorption of the FAs. In the triglycerides of human milk, the location of hexadecanoic acid,  $C_{16:0}$ , at the *sn*-2 position enhances the absorption of dietary FAs in the gut, as 2-monoglycerides. This information could assist in the design of lipids for infant formulae that are much more like human milk fat.

The composition of the milk phospholipids is also an important factor in the structure and transport of lipids in both human and bovine milk. The phospholipids are found predominately in the milk fat globule membrane.

These compounds originate from the membranes of the mammary cells that line ducts of the glands and are released with the milk during lactation. Their main function in the milk is as emulsifying agents and stabilizers of the milk fat globule membrane (Jensen *et al.*, 1990). They readily bind cations like calcium, sodium, and magnesium, and possibly interact with digestive enzymes. The subgroup of phospholipids known as gangliosides also act as antimicrobial agents and inhibit the action of enterotoxins from *Vibrio cholera* and *Esherichia coli*, even though they are present in only very small quantities. These lipids represent one of the many host defense mechanisms in human milk and, in part, explain why the incidence and overall severity of diarrhea is less in breast-fed infants than their formula-fed peers. The quantity of active ganglioside GM1 is much higher in human milk (12  $\mu\text{g/L}$ ) than in bovine milk (1  $\mu\text{g/L}$ ). Other trace phospholipids may also have as yet undiscovered non-nutritive roles.

The major sterol in both human and bovine milk is cholesterol. Trace amounts of other sterols are present also (e.g., lanosterol in bovine milk and desmosterol and some phytosterols in human milk). The amount of cholesterol present in human milk is 10–15  $\mu\text{g}/100\text{ml}$ , and although supplemented in some commercial formulae, it is present in amounts 5–10 times less than in human milk. Since the role of dietary cholesterol is still not fully defined, an intake similar to that obtained through breast-feeding is generally recommended (Uauy, 1990; Suskind and Lewinter-Suskind, 1993; Tsang *et al.*, 1993).

Dietary fat is also important as a source of the fat-soluble vitamins, A, D, E, and K. Requirements for the fat-soluble vitamins are several orders of magnitude lower than that of the essential fatty acids, being measured in micrograms rather than grams per day. Since the fat-soluble vitamins are required for growth and development, much interest has been focused on the concentration and availability of these vitamins in human milk.

Vitamin A (retinol) is found as such only in animal fats. Vegetables, such as dark green leaves and vegetable oils, like palm oil, contain precursors (pro-vitamin A), especially  $\beta$ -carotene, which the body converts to retinol (Gurr, 1994). As there are losses during the absorption and conversion of  $\beta$ -carotene to retinol, it is convenient to describe the vitamin A activity of foods in terms of retinol equivalents. The current RDA for infants up to 6 months of age is 375  $\mu\text{g}$  of vitamin A (retinol equivalents). Vitamin A deficiency that can lead to xerophthalmia, a condition that causes night blindness, and in the late stages, keratomalacia, is very rare in the developed world. However, it is a major problem elsewhere, affecting mostly children (Jensen *et al.*, 1992). While vitamin A is essential for proper development and function of the eyes, vitamin A taken in excess will accumulate in the liver, eventually leading to extensive liver damage and carotenemia.

Continued excessive intake of vitamin A may cause acute or chronic toxicity. Acute toxicity in children results from ingesting large doses ( $> 100\,000\ \mu\text{g}$  or  $300\,000\ \text{IU per diem}$ ); it is manifest as increased intracranial pressure and vomiting, which may lead to death unless ingestion is discontinued. Prognosis for recovery is excellent for adults and children; symptoms and signs usually disappear within 1–4 weeks after stopping vitamin A ingestion. However, prognosis for the fetus of a mother taking megadoses of vitamin A is guarded (Beers and Berkow, 2004).

The American Academy of Pediatrics recommends that exclusively breast-fed infants should receive vitamin D supplements to prevent rickets, a bone-weakening disease that physicians fear may be becoming more common. The Academy recommended that breast-fed infants should receive vitamin supplements beginning at 2 months of age and until they begin taking at least 500 ml daily of vitamin D-fortified milk. The Academy recommends multivitamin supplements containing 200 IU of vitamin D, available as over-the-counter liquid drops or tablets. Supplements containing only vitamin D, generally, are too concentrated to be safe for routine use by infants and must be supplied at recommended pediatric levels. The new recommendation also applies to infants who are not breast-fed but who do not drink at least 500 ml of fortified formula or milk daily and to children and adolescents who do not drink that much fortified milk, who are not regularly exposed to sunlight or who do not already take multiple vitamins with at least 200 IU of vitamin D (Hillman *et al.*, 1988).

Breast milk contains a small quantity of vitamin D and doctors used to think that babies could obtain adequate amounts if they also spent time in sunlight, which stimulates the body to produce vitamin D. However, growing concerns about skin cancer and recommendations that children wear sunscreen and avoid excessive exposure to the sun may put some children at risk of vitamin D deficiency and rickets. The new recommendations were prompted by reports of dozens of cases of rickets throughout the US in recent years (Gartner *et al.*, 2003).

The United States Centers for Disease Control and Prevention highlighted concerns about rickets in a 2001 report about several children hospitalized in the southern United States with the disease (Carvalho *et al.*, 2001). They included breast-fed infants who did not receive vitamin D supplements. Although reports are becoming more common, it is not clear if the actual incidence of rickets has increased since there are no national statistics on the condition. The most recent cases of rickets have been black children, whose skin does not absorb as much sunlight as white children. Children, who spend a lot of time indoors, perhaps because of parents' long working hours or safety concerns, also are at increased risk.

Like vitamin A, vitamin D is toxic if consumed in excess. Because cows' milk naturally contains a rather low level of vitamin D, it is the practice in several countries, notably the US, to fortify liquid milk and infant formulae with vitamin D (Haddad, 1992; Holick *et al.*, 1992).

Vitamin E is widespread in foods and is stored in the body so that deficiency states are very rare. A possible exception may be premature infants with very low fat stores. The concentration of  $\alpha$ -tocopherol in cows' milk ranges from 3.0 to 5.0 mg/L and is present at about the same level in human milk. While vitamin E has been shown to be essential for normal fertility in rats and other animals, it has never been proven to be necessary for human fertility. However, in recent years there has been renewed interest in the antioxidant function of vitamin E [e.g., in protecting the cardiovascular system (Sytkowski *et al.*, 1990; Gurr, 1994)].

$\alpha$ -Tocopherol is a powerful antioxidant and is present in the lipid bilayers of biological membranes, where it is thought to play a structural role. The products of peroxidation of unsaturated fatty acids can cause damage to the cells if the oxidative process is not limited and such damage appears to be exacerbated in animals given a diet deficient in vitamin E (Edwards-Webb and Gurr, 1988). These observations have led to the postulate that vitamin E plays a fundamental role in protecting the membrane fatty acids in living cells against oxidation.

Human milk contains about 2  $\mu$ g/L of vitamin K. Vitamin K denotes a group of compounds containing the 2-methyl-1,4-naphthoquinone chemical moiety. Phylloquinone is the plant form of the vitamin and is the most prevalent homologue in milk. The vitamin is required for the biosynthesis of prothrombin and many other essential blood-clotting factors (Jensen, 1992).

Analysis of the concentration of vitamin K in human milk by Canfield (1989, 1990) has shown that the amount of vitamin K in milk is not correlated with dietary intake. Further, human milk does not contain adequate vitamin K to prevent hemorrhagic disorders in newborns, in particular, pre-term infants with a very low liver store (Canfield *et al.*, 1991). This evidence suggests that vitamin K should be supplemented in all infants younger than six months of age.

However, even with these precautions, the American Academy of Pediatrics recommends that mothers should breast-feed their babies for at least a year, and longer if possible, while the World Health Organization says two years should be the minimum. Many studies have shown that babies who get human milk are healthier, less likely to become obese and may have better brain function (Gartner *et al.*, 1997). Infant milk manufacturers have taken note and regularly adjust their formulae to resemble human milk more closely.

### 14.3. Biological Benefits of Milk Fatty Acids

Absorption of dietary calcium is linked not only to the amount but also to the kind fatty acids in the diet of infants. In a controlled study, the fatty acid composition in one experimental formula was adjusted to simulate human milk, but the structure was dissimilar because the  $C_{16:0}$  in the formula was mostly at the *sn*-3 rather than the *sn*-2 position. The greatest loss of calcium in the feces occurred when formulae with the wrong proportions of  $C_{16:0}$  and  $C_{18:0}$  were fed. Fat absorption and calcium retention were highest in infants fed human milk, followed by infants fed formulae containing a high level of  $C_{12:0}$  (Nelson *et al.*, 1996, 1998).

The antimicrobial effects of fatty acids have been reported widely. Several intestinal parasites, including *Giardia lamblia*, the causative organism in giardiasis, and *Cryptosporidium*, the causative organism in cryptosporidiosis, both potentially lethal parasitic infections in infants, are killed by free fatty acids in the gut. Researchers have also reported antiviral activity of free fatty acids and possibly by monoglycerides produced by the action of lipoprotein lipase in both human and bovine milks (Isaacs *et al.*, 1990, 1992, 1995; Jensen *et al.*, 1992). Potassium oleate is particularly effective in this respect. The milk must be raw, as is almost always the case with human milk, but almost never with bovine milk. This antimicrobial effect is a potent host defense mechanism in breast-fed infants, particularly in third-world countries where it helps to control severe diarrhea, caused by these parasitic infections, and which are often lethal (Isaacs *et al.*, 1992, 1995).

### 14.4. Formulation of Infant Formula Using Milk Fat as an Ingredient

Both human and bovine milk contain about 3.5–4.0% fat emulsified as globules surrounded by a unique membrane, derived from apical plasma membrane of mammary epithelial cells (see Chapter 4). The outer layer contains glycoproteins and glycolipids, which form a lace-like network of glycoprotein filaments. The function of these filaments is unknown but they may enhance digestion and release triglyceride fatty acids by binding lipases. The filaments are lost on heating the milk. The human milk fat globule membrane is covered with thousands of microfilaments while the milk fat globule membranes of pasteurized bovine milk are relatively smooth (Jensen *et al.*, 1992). Both milks have about 0.5–0.6% of total lipid and 10–20 mg of phospholipid and cholesterol per 100 ml. Bovine milk contains more short-chain and saturated fatty acids, less  $C_{18:2}$ , and only small amounts of long-chain polyunsaturated fatty acids compared with human milk. The

triglyceride structure of both milks is unique in that the short-chain fatty acids are esterified mostly at the *sn*-3 position in bovine milk triglycerides, but much of the *sn*-2 fatty acids in human milk are C<sub>16:0</sub>. The triglycerides are metabolized differently (i.e., the short-chain acids are not absorbed *via* the intestinal micellar pathway).

The components in milk are an interrelated system in which compartmentalization is one of the factors controlling the flow of nutrients and metabolites to the infant. Human milk is not an inert multi-nutritional medium like a commercial formula, but should be seen as a fluid tissue, like blood. It contains live cells (i.e., macrophages, T and B lymphocytes, and polymorphonuclear leucocytes) and a wide variety of immunoglobulins, enzymes, vitamin-binding proteins, complement, nucleotides, and related nucleic acids, hormones and growth factors (Butte *et al.*, 1984; Garza *et al.*, 1987; Koldovsky, 1989; Franklin, 1989; Tsang *et al.*, 1993). Even the nutrient composition of human milk is not static as it is in commercial infant formulae, rather it varies considerably between individuals and during the course of lactation (Franklin, 1989). This changing composition of milk during lactation may reflect the changing nutritional needs of the full-term infant.

During the first 3 months, breast milk or formula is the only food an infant needs. Infant formula manufacturers attempt to mimic the composition of breast milk in their formulations. However, at present there is no scientific basis for simply deducing infant nutrient requirements from the composition of human milk. Moreover, absorption and metabolism of individual nutrients are not necessarily the same when the nutrients are supplied by human milk or when they are supplied by a formula. In those areas where we do not yet have sufficient data on the actual nutrient requirements of infants, the composition of human milk does give some guidance on acceptable intakes. Therefore, most infant formulae are designed to have the same protein, lipid, mineral, and vitamin content as human milk. The lipids in infant formulae are generally vegetable oils and therefore do not contain polyunsaturated fatty acids with more than 18 carbons. In contrast, both human milk and bovine milk contain long-chain polyunsaturated fatty acids (20–22 carbons) of both the  $\omega$ -6 and  $\omega$ -3 series, which are important for the development of human brain and retinal membranes. However, replacing natural dairy fats in milk-based infant formulae by vegetable oils appears to have other major draw-backs, including some non-reversible component interactions (i.e., protein-protein, protein-lipid, and lipid-mineral interactions) that may render some of the micronutrients in infant formula nutritionally unavailable (Hendricks *et al.*, 1994; Guo *et al.*,

1996). Mineral absorption studies that compared the absorption of the micro-nutrients, Cu, Fe, and Zn, from human milk *versus* experimental infant formulae confirmed that mineral uptake is more closely linked to the chemical interactions between the components in milk and formulae, than to mineral solubility alone (Glahn *et al.*, 1998; Hendricks *et al.*, 2001).

Development of infant formulae has focused on trying to match the composition of breast milk more closely. However, when trying to match the fatty acid content, careful consideration of the ingredients used is needed. Nelson *et al.* (1996, 1998) reported significantly lower fat and calcium absorption by healthy infants fed bovine milk protein-based infant formulae where the fat contained 53% or 45% of total fat as palm olein, a highly refined, triglyceride rich, bleached, and deodorized fraction of palm oil, compared to infants fed similar formulae without palm olein. The palm olein was included in infant formula fat blends to supply hexadecanoic acid at a similar level as in human milk. However, in human milk fat, hexadecanoic acid is primarily at the *sn*-2 position of the triglycerides, while in palm olein it is primarily at the *sn*-1 and *sn*-3 positions. It is now thought that this can have a profound effect on the absorption of the fatty acids and as a consequence, on calcium absorption (Ostrom *et al.*, 2002).

It is becoming more popular in the US for infant formula manufacturers to add fish oils to fortify infant formulae with long-chain polyunsaturated fatty acids, which are critical in early child development because they are necessary for the formation of neural tissues and cells of vascular tissue, but are produced *de novo* at very low levels from the dietary essential fatty acids C<sub>18:2</sub>,  $\omega$ -3 and C<sub>18:3</sub>,  $\omega$ -3. Typically, the long-chain fatty acids, docosahexaenoic acid (DHA; C<sub>22:6</sub>) and arachidonic acid (AA; C<sub>20:4</sub>), were not added to infant formulae available in the US until recently. Many commercial infant formulae manufacturers, including Wyeth, Ross and Mead Johnson, now produce infant formulae that are supplemented with DHA and AA. The level of DHA is approximately 0.32%, w/w of fat, and the level of AA is approximately 0.64% w/w of fat. Breast-milk naturally contains small amounts of these long-chain polyunsaturated fatty acids.

In a long-term feeding trial started in 1992 (Mercola, 2003), 111 newborns were fed a formula supplemented with DHA and AA and 126 were given a similar formula without DHA and AA. Another group of children in the study were breast-fed. When the children reached 6 years of age, their blood pressure was measured. Children fed the fatty acid-supplemented formula as infants had an average diastolic blood pressure that was three points lower than the control formula-fed group. The children who were exclusively breast-fed had the lowest diastolic blood pressure, but

it was not statistically different from the group that was given the supplemented formula. Since children with high blood pressure are likely to exhibit high blood pressure as adults, researchers noted that the consumption of DHA and AA in early life, preferably from breast-milk, may reduce the risk of cardiovascular disease in adulthood (Mercola, 2003).

It is also important to remember that approximately 60% of the human brain is composed of fatty material, 25% of which is DHA. Many companies are seeking United States Food and Drug Administration approval to add DHA to infant formulae. This would seem to be a wise choice considering the well-documented benefits of DHA in brain development. Although DHA is essential, if it is given out of balance, without EPA and AA as in the form being suggested by the FDA, it is potentially problematic. AA that is found in small amounts only in animal fats is also essential for brain development, a vital component of the cell membranes and a precursor of prostaglandins.

A fish oil supplement for infant formula has been shown to be effective at maintaining concentrations of  $\omega$ -3 long-chain polyunsaturated fatty acids in erythrocytes. Although human milk contains only small amounts of long-chain polyunsaturated fatty acids, it contains all the  $\omega$ -6 and  $\omega$ -3 fatty acids found in erythrocyte membranes. Carlson *et al.* (1987) demonstrated that if infant formula is supplemented with fish oil rich in EPA ( $C_{20:5}$ ,  $\omega$ -3) and DHA ( $C_{22:6}$ ,  $\omega$ -3), levels of these polyunsaturated fatty acids can be maintained post-birth, in erythrocyte membranes. These results indicate the effectiveness of providing long-chain polyunsaturated fatty acids directly in the diet rather than as precursors.

## 14.5. Summary

Lipids play a diverse role in human nutrition and development. They constitute a major energy source, provide an almost unlimited form of energy storage, and they act as vehicles for absorption and transport of fat-soluble vitamins (A, D, E, and K). Essential unsaturated fatty acids with a chain length of 18 carbon atoms with between two and six methylene-interrupted double bonds are critical for normal growth and development of the central nervous system, retina and vascular tissues throughout the body. Both the chemical structure of these fatty acids and their positions in the TGs present in milk (i.e., *sn*-1, *sn*-2 or *sn*-3), affect their rheological properties, melting point, crystallization behavior, and absorption. The phospholipids have an impact on the absorption of minerals from the gut and also act as anti-

crobial agents. Even the cholesterol in milk has a vital role in the formation of rapidly growing tissues and organs.

Milk fat is just one component in milk and is part of an interrelated system in which compartmentalization is one of the factors controlling the flow of nutrients and metabolites to the infant. Human milk is a multi-nutritional medium; a fluid tissue that provides the proper nutrients to the growing baby. The changing composition of breast milk during lactation might be geared to the changing nutritional needs of the full-term infant. Development of infant formulae must focus on trying to match the composition of human milk more closely. In this, it is important to note the significant role of milk fat as a natural component in the nutritional design of commercial infant formulae.

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# Lipolytic Enzymes and Hydrolytic Rancidity

H.C. Deeth and C.H. Fitz-Gerald

## Summary

Lipolysis, the enzymic hydrolysis of milk lipids to free fatty acids and partial glycerides, is a constant concern to the dairy industry because of the detrimental effects it can have on the flavor and other properties of milk and milk products. However, free fatty acids also contribute to the desirable flavor of milk and milk products when present at low concentrations and, in some cheeses, when present at high concentrations.

The enzymes responsible for the detrimental effects of lipolysis are of two main types: those indigenous to milk, and those of microbial origin. The major indigenous milk enzyme is lipoprotein lipase. It is active on the fat in natural milk fat globules only after their disruption by physical treatments or if certain blood serum lipoproteins are present. The major microbial lipases are produced by psychrotrophic bacteria. Many of these enzymes are heat stable and are particularly significant in stored products.

Human milk differs from cows' milk in that it contains two lipases, a lipoprotein lipase and a bile salt-stimulated lipase. The ability of the latter to cause considerable hydrolysis of ingested milk lipids has important nutritional implications.

## 15.1. Introduction

Hydrolytic rancidity in milk and milk products has been a concern to the dairy industry of most countries (Downey, 1975). Although it is not

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

considered a serious problem in many countries (IDF, 1983), the potential for problems exists at all times and, therefore, constant vigilance is necessary to ensure effective controls.

Hydrolytic rancidity results from the hydrolytic degradation of milk lipids. The hydrolysis is catalyzed by lipases and produces free fatty acids (FFAs), some of which have a low flavor threshold and can cause unpleasant flavors in milk and milk products. These flavors are variously described as rancid, butyric, bitter, unclean, soapy or astringent. The lipases involved are of two types: indigenous milk enzyme(s) and enzymes of microbial origin.

In the early 1900s, it was recognized that milk contains an enzyme capable of hydrolysing triglycerides and producing rancidity. Considerable research was carried out into the causes and effects of its action in milk. "Bitter milk of late lactation" and the increase in fat acidity following shaking, homogenization and certain temperature manipulations of raw milk were recognized as consequences of milk lipase action. It was also found that some developments in milking and processing methods (e.g., cold storage, mechanisation) could exacerbate the problem. For a review of this early work, see Herrington (1954).

During the 1950s and 1960s, studies focussed on the milk lipase system, the mechanism of its activation and the physico-chemical properties of the enzyme(s) involved. From these studies it was concluded that more than one lipase was present and several attempts to purify a milk lipase were reported. Jensen (1964) reviewed much of this work.

There were several new developments during the 1970s. Of particular importance was the purification and characterization of a lipoprotein lipase (LPL) and the acceptance of the postulate that this was the major, if not the only, lipase in cows' milk (Olivecrona, 1980). Similarly, the elucidation of the lipase system in human milk as consisting of an LPL and a bile salt-stimulated lipase, and the possible role of the latter in infant nutrition, were noteworthy (Fredrikzon *et al.*, 1978). Also, microbial lipolysis assumed substantial significance with the widespread use of low-temperature storage of raw milk and the recognition that heat-stable lipases produced by psychrotrophic bacteria were a major cause of flavor problems in stored dairy products (Law, 1979).

From the 1980s further advances in knowledge of the enzymes and the mechanism of their actions at the molecular level were made (Olivecrona *et al.*, 2003). Bacterial lipases received considerable attention (Fox *et al.*, 1989) and cloning of DNA encoding both milk lipases (Senda *et al.*, 1987; Nilsson *et al.*, 1990; Sbarra *et al.*, 1998) and bacterial lipases and esterases (Chung *et al.*, 1991; McKay *et al.*, 1995; Kojima *et al.*, 2003; Ro *et al.*, 2004) was achieved. Analytical methods for determining FFAs in milk and milk products with increased accuracy using high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC) were also developed

(Stead, 1989). Measurement of lipase activity has received considerable attention because of the defects caused in stored milk and dairy products by low levels of heat-resistant bacterial lipases (Deeth and Touch, 2000; Chen *et al.*, 2003)

In this chapter, the lipolytic enzymes in milk and milk products and the causes, consequences and assessment of their action are discussed. The significance of lipases in human nutrition and in the production of characteristic flavors in certain dairy products is also covered.

## 15.2. The Enzymes

### 15.2.1. Cows' Milk Lipase

Early research on lipolytic enzymes in cows' milk suggested that at least two major lipases were present: a "plasma lipase" in the skim portion and a "membrane lipase" associated with the milk fat globule membrane (Tarassuk and Frankel, 1957) while later research indicated that there might be up to six different molecular species with lipase activity (Downey and Andrews, 1969). However, work by Korn (1962) showed that milk contained a lipoprotein lipase (EC 3.1.1.34) (LPL) with properties very similar to those of post-heparin plasma, adipose tissue and heart LPLs, particularly the enhancement of its activity on emulsified triglycerides by blood serum lipoproteins. It is now accepted that LPL is the major, if not the only, lipase in cows' milk. Its properties have been reviewed by Olivecrona *et al.* (2003).

LPL is synthesized in the mammary gland secretory cells and most is transported to the capillary endothelium where it hydrolyzes triglycerides in circulating lipoproteins to FFAs and 2-monoglycerides. These products are absorbed by the mammary gland and used for the synthesis of milk fat. The LPL in milk appears to be identical with the enzyme in the mammary gland (Askew *et al.*, 1970) and to be the result of a spillover. Its level in milk is low at parturition but increases rapidly during the first few days of lactation and remains almost constant for the remainder of the lactation (Saito and Kim, 1995).

Under normal circumstances, most of the LPL in milk is in the skim milk fraction and the major part of this is associated with the casein micelles (Fox *et al.*, 1967). Some is in soluble form (Anderson, 1982a) and a small amount is associated with the milk fat globule membrane (Deeth and Fitzgerald, 1975a). The enzyme is bound to the caseins principally by electrostatic interactions; NaCl (0.75–1M) releases most of it into the serum phase where it is associated with casein in aggregates of molecular weight of *ca.* 500 000 Da (Hoynes and Downey, 1973). The electrostatic binding of lipase in the micelle appears to be *via* positive charges on the enzyme to negatively

charged caseins, (e.g.,  $\kappa$ -casein) (Downey and Murphy, 1975). LPL binds strongly to negatively charged heparin, enabling it to be dissociated from the casein micelle by low concentrations of sodium heparin (5  $\mu\text{g/l}$ ) (Hoynes and Downey, 1973). Hydrophobic associations may also be involved in the lipase-casein interaction since the lipase can be dissociated from the complex by dimethylformamide (Fox *et al.*, 1967).

LPL is a glycoprotein (8% by weight carbohydrate) with a native molecular weight of around 100 000 Da and a monomer subunit of about 50 000 Da (Kinnunen *et al.*, 1976). Senda *et al.* (1987) calculated the molecular weight of the unglycosylated form as 50 548 Da based on the cDNA encoding it. LPL has a serine at the active site, located in a beta turn in the enzyme, similar to that at the active site of other serine hydrolases (Reddy *et al.*, 1986).

LPL is a relatively unstable enzyme, being inactivated by ultraviolet light, heat, acid, oxidising agents (Frankel and Tarassuk, 1959), and prolonged freezing (Needs, 1992). Even in the mammary gland at body temperature, it is inactivated slowly and, as a consequence, milk contains a mixture of active and inactive LPL (Olivecrona and Bengtsson-Olivecrona, 1991). In milk, it is believed that LPL is stabilized by a factor in the skim milk fraction (Posner and Bermúdez, 1977), possibly a heparin-like glycosaminoglycan (Iverius *et al.*, 1972). Caseins (Lebedev and Umanskii, 1979; Anderson, 1982b; Kim *et al.*, 1994) and some lipids (Shimada *et al.*, 1982) also stabilize it.

High-temperature short-time (HTST) treatment ( $72^\circ\text{C} \times 15\text{ s}$ ) of milk almost completely inactivates the enzyme (Luhtala and Antila, 1968; Andrews *et al.*, 1987; Farkye *et al.*, 1995) so that little if any lipolysis caused by milk lipase occurs in pasteurised milk (Downey, 1974). Somewhat higher temperatures are required for cream pasteurization because of the protective effect of the fat (Nilsson and Willart, 1961; Downey and Andrews, 1966). However, some workers have reported that a more severe heat treatment, [e.g.,  $79^\circ\text{C} \times 20\text{ s}$ , (Shipe and Senyk, 1981) or  $85^\circ\text{C} \times 10\text{ s}$  (Driessen, 1987)] is required to inactivate completely milk lipase.

The normal substrates for LPLs are long-chain triglycerides in blood chylomicrons and lipoproteins. These particles contain the apolipoproteins (e.g., apo-LP CII), which activate the enzyme (Östlund-Lindqvist and Iverius, 1975). Blood serum, either cows' or human, has an overall activating effect in assays of LPL using emulsified long-chain triglyceride substrates (Egelrud and Olivecrona, 1973). In such assays, a fatty acid acceptor such as bovine serum albumin (BSA) is required because LPL is susceptible to product inhibition by FFAs, which accumulate at the lipid-water interface (Bengtsson and Olivecrona, 1980). LPL is also active on tributyrin, but in this case requires neither serum co-factors nor fatty acid acceptors (Rapp

and Olivecrona, 1978) and a catalytic rate of about 50% of its "lipoprotein lipase activity" (measured on serum-activated long-chain triglycerides) is observed (Egelrud and Olivecrona, 1973). *p*-Nitrophenyl esters (Shirai and Jackson, 1982), Tween 20 and monoglycerides are also hydrolyzed in the absence of serum co-factors (Egelrud and Olivecrona, 1973).

In milk, LPL is not normally active on milk fat because of the protection afforded the fat by the milk fat globule membrane. However, addition of blood serum facilitates the interaction between the enzyme and the fat globule and lipolysis ensues (Castberg and Solberg, 1974; Jellema and Schipper, 1975). The mechanism of this serum-mediated induction of lipolysis is not known, although Bengtsson and Olivecrona (1982) concluded that the activating apo-LP CII performed a dual role of enhancing both binding of LPL to the fat globule and its catalytic efficiency.

Phospholipids also have a role in the LPL-catalyzed hydrolysis of triglycerides. The activator apo-LPs exhibit enhanced activation in the presence of phospholipids such as phosphatidyl choline (La Rosa *et al.*, 1970; Blaton *et al.*, 1974) and in milk there is evidence that apo-LPs in the absence of phospholipids are unable to initiate lipolysis of intact milk fat globules by the indigenous LPL (Driessen and Stadhouders, 1974; Clegg, 1980). The phospholipids are believed to be involved in the reaction through their interaction with the substrate rather than with the enzyme (Blaton *et al.*, 1974).

The discovery of lipolysis-inhibiting glycoproteins in skim milk, particularly proteose-peptone fraction 3 (PP3) (Anderson, 1981; Cartier *et al.*, 1990; Girardet *et al.*, 1993), and in the milk fat globule membrane (Shimizu *et al.*, 1982; Kester and Brunner, 1982; Sundheim and Bengtsson-Olivecrona, 1987b) supports an earlier observation (Dunkley and Smith, 1951) that milk contains lipolysis-inhibiting factors. PP3, a phosphorylated glycoprotein with an apparent molecular mass of 28 000 Da and 135 amino acid residues (Sørensen and Petersen, 1993), has been reviewed by Girardet and Linden (1996). Of particular significance is a C-terminal 38-amino acid residue segment, which binds to membranes (Bak *et al.*, 2000). Shimizu and Yamauchi (1983) considered PP3 to be identical with a major glycoprotein in the milk fat globule membrane, which can be solubilized with dilute NaCl.

Phosphatidyl choline is also hydrolyzed by milk LPL in the presence of serum co-factors (Stocks and Galton, 1980) to yield FFAs and lysophosphatidyl choline (Scow and Egelrud, 1976). The importance of this function of LPL appears to be in facilitating its access to the triglyceride core of particles having a phospholipid-containing membrane. The lysophospholipids have a high affinity for both LPL and lipoproteins (Portman and Alexander, 1976) and are powerful membrane-perturbing agents (Weltzien, 1979) and may aid lipolysis of milk fat in its globular form (Bläckberg *et al.*, 1981a).

LPL exhibits no fatty acid specificity during hydrolysis of mixed triglycerides but does have strong positional specificity (Morley and Kuksis, 1977). It acts on primary ester bonds with some preference for the *sn*-1 over the *sn*-3 position of triglycerides (Somerharju *et al.*, 1978) and can hydrolyze 2-monoglycerides only after their conversion to the *sn*-1 or *sn*-3 isomers (Nilsson-Ehle *et al.*, 1973). It shows phospholipase A<sub>1</sub> activity on phosphatidyl choline (i.e., it hydrolyzes the primary ester bond at the *sn*-1 position). This contrasts with most phospholipases A, which exhibit A<sub>2</sub> activity.

### 15.2.2. Human Milk Lipases

Human milk differs from cows' milk in that it contains, in addition to an LPL similar to that in cows' milk, a bile salt-stimulated lipase (BSSL), which appears to have no counterpart in cows' milk (Hernell and Blackberg, 1994). In addition, a third lipase known as platelet-activating factor acetylhydrolase activity (PAF-AH) has been demonstrated in human milk; the activity is absent, or extremely low in cows' milk (Furukawa, *et al.*, 1994).

The LPL in human milk resembles most other serum-stimulated lipases. While it is capable of hydrolysing triglycerides in the absence of exogenous serum factors, its activity is increased several-fold by blood serum (Hernell and Olivecrona, 1974a). LPL occurs mostly in the skim milk phase of human milk but after freeze-thawing is mostly associated with the cream phase (Neville *et al.*, 1991). In general, human milk contains less than half the LPL activity found in cows' milk (Hernell and Olivecrona, 1974a) but can become rancid on cold storage (Dill *et al.*, 1984; Hamosh *et al.*, 1996) or frozen storage at  $\leq -20^{\circ}\text{C}$  (Berkow *et al.*, 1984); freezing and thawing increase the extent of lipolysis. Ultrasonic homogenization at  $< 45^{\circ}\text{C}$  stimulates lipolysis but at  $> 55^{\circ}\text{C}$  it inactivates the lipolytic activity (Martinez *et al.*, 1992). In contrast to the case of cows' milk (Section 3.2), "spontaneous lipolysis" in human milk is strongly correlated ( $r = +0.90$ ) with the LPL activity of the milk (Castberg and Hernell, 1975).

Human milk is unusual in containing a lipase that is activated by bile salts. BSSL has been found in the milk of only a few other mammals: gorilla (Freudenberg, 1966), cat, and dog, (Freed *et al.*, 1986), and ferret (Ellis and Hamosh, 1992). Ferret milk contains up to 20 times as much BSSL as human milk and constitutes a significant proportion (1–2%) of the total milk protein. BSSL in human milk is present in multimolecular forms, which differ from each other by the extent or quantity of glycosylation in the proline-rich region of the C-terminus of the enzyme (McKillop *et al.*, 2004).

Human BSSL shows immunological identity with the carboxyl ester hydrolase in pancreatic juice, and the two enzymes are very similar in molecular and kinetic properties (Bläckberg *et al.*, 1981b). In fact, Nilsson

*et al.* (1990) have shown from molecular cloning experiments that the two enzymes are identical and encoded by the same gene. However, milk BSSL is, evidently, synthesized in the lactating mammary gland (Bläckberg *et al.*, 1987).

BSSL is located mainly in the milk serum and is optimally active at around pH 8 and 37°C in the presence of 8–14 mM sodium taurocholate. In the absence of bile salts, it can hydrolyze soluble esters (e.g., *p*-nitrophenyl acetate) and tributyrin, but is inactive against high molecular weight triglycerides. Bile salts promote the lipolysis of emulsified and micellar substrates, such as triolein and milk fat, and also enhance activity on emulsified tributyrin and soluble esters (Jubelin and Boyer, 1972; Hernell and Olivecrona, 1974b; Hall and Muller, 1982). Unlike LPL, BSSL has no positional specificity, hydrolysing triglyceride to mainly FFA and glycerol, but has a preference for short-chain and polyunsaturated fatty acids (Wang *et al.*, 1983; Wang, 1991).

The activation of BSSL is specific to primary bile salts. Bile salts, secondary as well as primary, protect BSSL against inactivation by intestinal proteinases. BSSL is inactivated by heating at 50°C for 1 h, but sodium taurocholate prevents loss of activity. The enzyme is stable in buffer for 1 h at 37°C between pH 3.5 and 9. It is inhibited by blood serum, 1M NaCl, protamine sulphate, eserine and diisopropylfluorophosphate (Hernell, 1975; Bläckberg and Hernell, 1983).

BSSL is present in high amounts in pre-term and mature milks, and varies little throughout lactation, with activity in the order of 100 times greater than LPL in human and cows' milks (Hernell and Olivecrona, 1974a; Freed *et al.*, 1987). It plays an important role in digestion in infants (O'Connor and Cleverly, 1989) and may provide a defence mechanism against parasites such as *Giardia lamblia* (Reiner *et al.*, 1986; Gillin *et al.*, 1991) (Section 5.2).

### 15.2.3. Milk Lipases of Other Species

Lipase activity has been detected in the milk of many other species and, where characterized, is generally serum-stimulated (e.g., goat, buffalo, horse, guinea pig, rat, rabbit, cat, and dog) (Hamosh and Scow, 1971; Jensen and Pitas, 1976; Freed *et al.*, 1986). With few exceptions, LPL appears to be the only significant indigenous lipase in milk. BSSL occurs in the milk of a small number of species (Section 2.2).

Goats' milk LPL has been studied extensively. In contrast to cows' milk LPL, it is distributed approximately equally between the cream and serum phases, with a small amount (*ca.* 10%) attached to caseins. Total activity is much lower than in cows' milk, and varies widely between individuals

(Chilliard *et al.*, 1984). Between-breed and within-breed genetic effects on activity appear to exist (Chilliard, 1982). Physiological variations, such as stage of lactation, also influence activity (Chilliard and Morand-Fehr, 1978). Two distinct LPL forms with molecular weights in the range 55 000–66 000 Da have been isolated and found to differ from cows' milk LPL (De Feo *et al.*, 1982).

Like cows' milk LPL, goats' milk LPL is inactivated by pasteurization. However, it is not inactivated by high-pressure treatment (500 MPa, 15 min, 20°C) (Trujillo *et al.*, 1999) and appears to contribute to lipolysis in cheese made from pressure-treated milk (Buffa *et al.*, 2001).

As with cows' milk (Section 3.1), homogenization, agitation (e.g., during machine milking) and temperature manipulation (cooling–warming–cooling) can initiate lipolysis in goats' milk (Bjørke and Castberg, 1976; Chilliard and Morand-Fehr, 1976; Morand-Fehr *et al.*, 1990). Spontaneous lipolysis also occurs in the milk of some goats. In contrast to cows' milk, there is a significant correlation between this lipolysis and LPL activity, perhaps because of the relatively high proportion of LPL associated with the fat globules. The influence of inhibiting and activating factors is also apparent (Chilliard *et al.*, 1984). The proteose peptone fraction of goats' milk inhibits mechanically induced and blood serum-activated lipolysis in goats' milk (Arora and Joshi, 1994). Lipolysis is linked with the occurrence of a "goaty" flavor in the milk (Bjørke and Castberg, 1976; Brendehaug and Abrahamsen, 1986).

Buffaloes' milk contains an LPL similar to cows' milk LPL and in comparable quantities. A higher proportion is located in the cream (e.g., 23% compared to 12% for cows' milk; Balasubramanya *et al.*, 1988). Bhavadasan *et al.* (1988) found no relationship between the extent of lipolysis and LPL activity in either species. Lipolysis by LPL is inhibited by proteose peptone fractions 3, 5 and 8 from buffaloes' milk, with the PP3 fraction being the most inhibitory (Ram and Joshi, 1989). As in cows' milk, lipolysis can be induced by shaking or homogenization (Sammanwar and Ganguli, 1974).

Guinea pigs' milk contains a high level of LPL (20–50-fold that of cows' milk), with more than 90% in the skim (Hamosh and Scow, 1971). Guinea pig LPL has been purified and found to be very similar in molecular structure and properties to the LPLs of cows' and human milks (Wallinder *et al.*, 1982; Bengtsson-Olivecrona *et al.*, 1986).

LPL activity in rats' milk is only about 0.2% of that of guinea pigs' milk, probably reflecting interspecial differences in milk secretory processes (Hamosh and Scow, 1971). Activity in dogs' and cats' milk is also low, of a similar order to human milk (Freed *et al.*, 1986).

cDNAs have been cloned for guinea pig (Enerbäck *et al.*, 1987) and mouse LPL (Kirchgessner *et al.*, 1987). Deduced amino acid sequences show considerable homology with human and bovine LPLs and with hepatic and pancreatic lipases, suggesting a common ancestral lipase gene (Olivecrona and Bengtsson-Olivecrona, 1991).

#### 15.2.4. Esterases of Cows' Milk

In addition to the now well-documented lipase system, cows' milk contains several other carboxyl ester hydrolases, collectively referred to as esterases. These are distinguished from lipases by their ability to act on ester substrates in solution rather than in an emulsified form (Jaeger *et al.*, 1994) and/or by their preference for hydrolysing esters of short-chain rather than long-chain acids (Okuda and Fujii, 1968).

Although several reports concerning esterases in milk have appeared in the literature, little detailed information on the individual enzymes is available. Arylesterase or A-esterase (EC 3.1.1.2), carboxylesterase or B-esterase (EC 3.1.1.1), and cholinesterase or C-esterase (EC 3.1.1.7; EC 3.1.1.8) have been identified (Forster *et al.*, 1961; Kitchen, 1971; Nakanishi and Tagata, 1972; Deeth, 1978).

Arylesterase has received considerable attention because of its elevated level in colostrum and mastitic milk (Forster *et al.*, 1959; Marquardt and Forster, 1965). Since its level in mastitic milk correlates well with other indices of mastitis (Luedecke, 1964), it has been suggested as a sensitive indicator of the disease (Forster *et al.*, 1961; Downey, 1974). The enzyme is believed to originate from blood, where its activity is up to 2000 times that in milk (Marquardt and Forster, 1965).

Carboxylesterase activity is elevated in mastitic milk and colostrum (Fitz-Gerald *et al.*, 1981) and may correspond to that of the reported "lipases" from somatic cells (Gaffney and Harper, 1965; Azzara and Dimick, 1985a) and colostrum (Driessen, 1976), respectively. The retinyl esterase activity that co-purifies with, but can be separated from, LPL may also be due to a carboxylesterase (Goldberg *et al.*, 1986). It is of interest that the BSSL in human milk that has been shown to be identical with pancreatic carboxylesterase, has retinyl esterase activity (O'Connor and Cleverly, 1989).

Compared with the total lipase activity on emulsified milk fat or tributyrin (0.25–2.5  $\mu\text{mol/ml/min}$ ), the esterase activity (on soluble tributyrin) is quite low, about one tenth (Downey, 1974). This may not be so for some abnormal milks where esterase levels are markedly elevated [10–12 times (Marquardt and Forster, 1962) and up to 37 times (Deeth, 1978)]. The significance of these esterases in cows' milk and their relationship to each other, to LPL, and to esterases of other tissues remain to be determined.

### 15.2.5. Lipases of Psychrotrophic Bacteria

Extracellular lipases produced by psychrotrophic bacteria have considerable potential for causing hydrolytic rancidity in milk and milk products. The bacteria principally responsible for these lipases are pseudomonads, particularly *Pseudomonas fluorescens*, and *P. fragi*, Enterobacteriaceae such as *Serratia*, and *Acinetobacter* spp. Other significant organisms include *Achromobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Micrococcus*, and *Moraxella* (Stewart *et al.*, 1975; Muir *et al.*, 1979; Shelley *et al.*, 1987; Abdou, 2003). In a study of the lipolytic flora of raw milks showing lipolytic defects, Shelley *et al.* (1987) found *P. fluorescens* to be the species most frequently encountered but *P. fragi* to be associated with the most severe lipolytic defects. Reviews concerning bacterial lipases and their significance in milk and other foods have been published by Cogan (1977), Law (1979), Stead (1986), and Sørhaug and Stepaniak (1997), and by several authors in “*Enzymes of Psychrotrophs in Raw Food*” edited by McKellar (1989).

While many authors have reported that *Pseudomonas* species, particularly *P. fluorescens*, are the dominant lipase-producing psychrotrophs in milk, there appears to be considerable differences in lipase production among these closely related species (Wang and Jayarao, 2001; Deeth *et al.*, 2002). Dogan and Boor (2003) reported that patterns of extracellular enzyme activity among *Pseudomonas* isolates appeared to be associated with ribotypes, with ribotypes 50-S-8 and 72-S-3 producing the highest extracellular lipase activity. Ribotyping has been used to identify high lipase-producing ribotypes. However, Wang and Jayarao (2001) found that although the 16S-23S PCR ribotyping technique allowed differentiation between strains, it did not concur with the API 20 NE biotypes and the lipolytic profiles. They concluded that use of biotypes in conjunction with lipolytic (and proteolytic) profiles might have practical value for conducting trace-back studies related to *P. fluorescens*.

Lipases are produced by psychrotrophic bacteria during the late log and early stationary phases of growth, often reaching a peak and then decreasing (Stead, 1987; Stepaniak *et al.*, 1987b; Griffiths, 1989; Rowe *et al.*, 1990). Little lipase is produced before cell numbers reach  $10^6$ – $10^7$ /ml. The rate of growth of does not appear to correlate well with lipase production. In fact, Stevenson *et al.* (2003) found that, in most cases, pseudomonads from pasteurized milk with the lowest growth rate produced lipase earlier than those with a high growth rate. The influence of a wide variety of environmental and nutritional factors on the production of these enzymes has been reviewed in McKellar (1989).

Pseudomonad species usually constitute the largest percentage of lipolytic psychrotrophs in raw milk and cream and hence have attracted most attention. Lipases have been purified from *P. fluorescens* (Sugiura *et al.*,

1977; Severina and Bashkatova, 1979; Andersson, 1980; Dring and Fox, 1983; Stepaniak *et al.*, 1987a), and *P. fragi* (Lawrence *et al.*, 1967; Lu and Liska, 1969). The isolation and molecular characteristics of lipases from psychrotrophic bacteria have been reviewed by Fox *et al.* (1989).

In general, these microbial lipases have a molecular weight in the region 25 000 to 50 000 Da. Sugiura *et al.* (1977) purified a lipase from *P. fluorescens* with molecular weight 33 000 Da and found it to be a single polypeptide chain with no lipid or carbohydrate and without disulphide linkages, while Dring and Fox (1983) and Stepaniak *et al.* (1987a) isolated *P. fluorescens* lipases under dissociating conditions with a molecular weight of around 16 000 Da. On the basis of known amino acid sequences of lipases from *Pseudomonas* and *Burkholderia*, Dieckelmann *et al.* (1998) concluded that there were two major lipase groups, one of molecular weight approximately 30 kDa comprising lipases from *P. fragi*, *P. aeruginosa*, *P. fluorescens* C9 and *Burkholderia*, and one of approximately 50 kDa comprising *P. fluorescens* lipases. However, some workers have found lipase activity associated with material of molecular weight >100 000 Da, which probably represents aggregates of subunits as the enzyme associates strongly (Dring and Fox, 1983; Stepaniak *et al.*, 1987a); the lipase from *P. fluorescens* SIK W1 has a molecular weight of 52 000 Da by SDS polyacrylamide gel electrophoresis or 48 179 Da as predicted from the isolated gene encoding it. This enzyme contains nine cysteine residues, which may participate in disulphide bridges (Chung *et al.*, 1991). A gene coding for the lipase of *P. fragi* that was cloned into *E. coli* had a nucleotide sequence corresponding to a protein of 135 amino acids with a predicted molecular weight of 14 643 Da (Kugimiya *et al.*, 1986).

The pH optimum of the lipases is usually in the alkaline region between 7 and 9. They generally show highest activity at 40–50°C, although there are reports of higher or lower temperature optima (Stead, 1986). The apparent optimum temperature may change with the state of purity of the enzyme (Severina and Bashkatova, 1979) and with the assay conditions used (Fitz-Gerald and Deeth, 1983). Many of these lipases show activity at the low temperatures used for storage of dairy products, [e.g., 10°C (Te Whaiti and Fryer, 1978), 1°C (Landaas and Solberg, 1978), and –10°C (Nashif and Nelson, 1953)].

One of the most important properties of these lipases is their heat stability (Stepaniak *et al.*, 1995). This varies with the species and strain (Fitz-Gerald *et al.*, 1982) and also with the medium in which they are heated (Andersson *et al.*, 1979). Many are sufficiently stable to retain at least some activity after pasteurization (Law *et al.*, 1976; Fitz-Gerald *et al.*, 1982; Kalogridou-Vassiliadou, 1984), and even after UHT treatment (Kishonti, 1975; Mottar, 1981; Christen *et al.*, 1986). Milk proteins, except  $\kappa$ -casein,

have been shown to have a thermostabilising effect on these lipases (Kumura *et al.*, 1993). For example, heating a lipase with  $\beta$ -lactoglobulin at 80–90°C caused virtually no change in enzyme activity. Some workers have reported a two-stage inactivation on heating, an initial rapid loss of activity, followed by a slow or even negligible decline (Fox and Stepaniak, 1983; Swaisgood and Bozoğlu, 1984; Driessen, 1987). A recent report suggests that high intensity pulsed electric field treatment may be more effective in inactivating lipases from *P. fluorescens* than thermal treatments; treatment with 80 pulses at 27.4 kV/cm in batch mode caused over 60% inactivation (Bendicho *et al.*, 2002). Similarly, sonication under pressure (650 kPa) at an elevated temperature (110 or 140°C) (manothermosonication) has also been found to inactivate a *P. fluorescens* lipase more efficiently than the corresponding heat treatment alone (Vercet *et al.*, 1997).

Some of the lipases are less stable at temperatures  $\leq$  ca. 70°C than at higher temperatures (Kroll, 1989) and are thus susceptible to 'low-temperature inactivation' (LTI), which is effective for inactivating the corresponding proteinases (e.g., 55°C for 1 hour; Barach *et al.*, 1976). However, in fat-containing media, considerable lipolysis can occur during prolonged heating at 55°C and hence treatment at this temperature may have limited value for eliminating these lipases from milk products (Griffiths *et al.*, 1981; Fitz-Gerald *et al.*, 1982). Heating at a temperature  $>70^\circ\text{C}$  (up to ca. 120°C) can cause activation of some lipases (Andersson *et al.*, 1979; Fitz-Gerald *et al.*, 1982) and exacerbate lipolysis problems. Bucky *et al.* (1987) combined UHT treatment with a subsequent LTI treatment of 60°C for 5 min in a patented process, which considerably enhances the effectiveness of UHT treatment in reducing lipase activity. Investigations of the mechanism of heat inactivation of these enzymes have concluded that inactivation at high or low temperatures involves different denatured states of the enzyme (Swaisgood and Bozoğlu, 1984) and that proteinases in the environment do not cause LTI, although lipases may exhibit reduced stability in their presence (Kumura *et al.*, 1991).

Unlike the corresponding proteinases, the lipases do not appear to contain metal ions (Nadkarni, 1971; Sugiura and Oikawa, 1977), but do require metal ions, such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , for activity (Severina and Bashkatova, 1979; Fitz-Gerald and Deeth, 1983). Excess EDTA causes complete inhibition of most bacterial lipases, which can be reversed by  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . *Acinetobacter* lipases have been found to be irreversibly inactivated by EDTA, while *P. aeruginosa* lipases are exceptional in being almost unaffected by excess EDTA (Nadkarni, 1971; Fitz-Gerald and Deeth, 1983). Some heavy metals are inhibitory, in particular zinc, iron, mercury, nickel, copper, and cobalt (Fitz-Gerald and Deeth, 1983; Fox *et al.*, 1989). These metals are effective at concentrations of less than 10 mM.

A low level of NaCl (10 mM) may cause activation (Khan *et al.*, 1967), although a high concentration inhibits the lipases. However, more than half of the activity remains in the presence of NaCl (2 M), a level similar to that in the aqueous phase of salted butter (Fitz-Gerald and Deeth, 1983).

Unlike milk LPL, microbial lipases do not require a fatty acid acceptor such as BSA (Bengtsson and Olivecrona, 1980). Blood serum has been found to activate some of these enzymes (Fitz-Gerald and Deeth, 1983), including *P. fluorescens* lipases, and these have, consequently, been designated lipoprotein lipases (Aisaka and Tarada, 1979; Stepaniak and Sørhaug, 1989).

While psychrotrophs produce true lipases that act on emulsified triglyceride substrates, many also produce esterases, which prefer soluble substrates (Stewart *et al.*, 1975; O'Donnell, 1978) or short-chain triglycerides, such as tributyrin, to long-chain triglycerides (Muir *et al.*, 1979; Garcia *et al.*, 1989). Chung *et al.* (1991) elegantly demonstrated that the esterase and lipase activities of a lipolytic strain of *P. fluorescens* were due to two different enzymes. After inserting fragments of the pseudomonad DNA into *E. coli* JM83, 20 of the resulting 12 000 recombinant colonies exhibited activity on tributyrin but only one had activity on longer chain triglycerides (in olive oil). Similarly, McKay *et al.* (1995) constructed highly lipolytic strains of *P. fluorescens* that over-produced, or were specifically deficient in, a lipase (encoded by *lipA*) and an esterase (encoded by *estA*). Analysis of broth cultures showed that the lipase was secreted into the culture medium while the esterase was intracellular and not secreted. They concluded that production of FFAs in milk cultures by this organism was due solely to the single secreted lipase and that the esterase was not involved. They also made the interesting observation that the profile and level of the FFAs accumulated in milk cultures was the result of both the production and degradation of FFA. This may explain the contradictory reports in the literature on the FFA profile in products contaminated with lipolytic organisms.

Lawrence *et al.* (1967) reported some preference for long-chain triglycerides by a *P. fragi* lipase but for short-chain triglycerides by a lipase from *Micrococcus freudenreichii*. Temperature may have an influence on the apparent specificity of lipolysis, with relatively more short-chain and unsaturated fatty acids being released from milk fat at lower temperatures. This appears to be a reflection of the physical state of the substrate (Alford and Pierce, 1961; Sugiura and Isobe, 1975).

Purified *P. fluorescens* lipases have activity against natural vegetable oils and a range of synthetic triglycerides from tributyrin to triolein (Severina and Bashkatova, 1979), and show preference for triglycerides rather than monoglycerides, and for medium-chain-length substrates (i.e., containing C<sub>8</sub>–C<sub>10</sub> fatty acids) (Bozoğlu *et al.*, 1984). Most of the lipases have a preference for the primary (*sn*-1 and *sn*-3) positions of triglycerides (Alford

*et al.*, 1964; Nadkarni, 1971; Cooke, 1973). The profile of FFAs released from a given fat may be characteristic of the enzyme (Woo and Lindsay, 1983b; Ren *et al.*, 1988).

The lipases produced in crude cultures are usually capable of hydrolysing the triglyceride in intact milk fat globules (Fitz-Gerald and Deeth 1983), a property not exhibited by the indigenous milk LPL because of the protection afforded by the milk fat globule membrane (Danthine *et al.*, 2000). It is not known whether the lipases *per se* can penetrate the milk fat globule membrane or whether the membrane is first disrupted by other enzymes such as glycosidases, proteases and phospholipases (Mabbitt, 1981; Marin *et al.*, 1984; Alkanhal *et al.*, 1985; Cousin, 1989). Griffiths (1983) showed that a phospholipase C of *Bacillus cereus* increased the lipolytic activity of indigenous LPL in raw milk by making the substrate more susceptible to hydrolysis. However, it did not enhance the activity of lipase from a fluorescent pseudomonad. He considered that addition of phospholipase C had the same effect on lipolysis by lipoprotein lipase as homogenization of raw milk (Section 15.3.1 2).

### 15.2.6. Phospholipases

Phospholipases are potentially important in milk and milk products because of their ability to degrade the phospholipids of the milk fat globule membrane, thereby increasing the susceptibility of the milk fat to lipolytic attack (Fox *et al.*, 1976; Griffiths 1983).

Cows' milk LPL has phospholipase A<sub>1</sub> activity (Scow and Egelrud, 1976), but its action on milk phospholipids has not been recorded. Freshly secreted goats' milk has been shown to have phospholipase A activity (Long and Patton, 1978) but it is not known whether this can be attributed to the LPL of that milk. Human milk contains an acid sphingomyelinase C, as well as ceramidase activity provided by the bile salt-stimulated lipase present (Nyberg *et al.*, 1998).

Several psychrotrophic bacteria produce extracellular phospholipases, the most prevalent in milk being pseudomonads (particularly *P. fluorescens*), *Alcaligenes*, *Acinetobacter*, and *Bacillus* species (Fox *et al.*, 1976; Owens, 1978a; Phillips *et al.*, 1981). Most of these produce phospholipase C, some produce phospholipase A<sub>1</sub> and some produce both types (Deeth, 1983). *Serratia* spp. have been shown to produce only phospholipase A (Deeth, 1983), while *P. fragi* has been reported not to produce phospholipases (Kwan and Skura, 1985). Phospholipase C from some pseudomonads has been purified and characterised (Doi and Nojima, 1971; Sonoki and Ikezawa, 1975; Stepaniak *et al.*, 1987a; Ivanov *et al.*, 1996). Like the lipases, many of these enzymes have considerable heat stability and are not destroyed by pasteurization

(Owens, 1978b; Stepaniak *et al.*, 1987a; Koka and Weimer, 2001). Their heat stability varies with the bacterial strain and growth conditions (Koka and Weimer, 2001)

The phospholipases of *Bacillus* spp., especially *B. cereus*, have been studied because of their association with the “bitty cream” or “broken cream” defect in milk (Stone, 1952). By partially degrading the milk fat globule membrane, they initiate agglutination of the fat globules into cream flakes or flecks (Labots and Galesloot, 1959). The degradation is caused by phospholipase C (Shimizu *et al.*, 1980), although *B. cereus* produces a sphingomyelinase (Ikezawa *et al.*, 1978), which may be involved also. Phospholipase-producing bacteria other than *Bacillus* species do not appear to cause “bitty cream” (Labots and Galesloot, 1959; Owens, 1978a). The phospholipases of *B. cereus* have been isolated, purified, and extensively characterised (Zwaal *et al.*, 1971; Ikezawa *et al.*, 1978).

### 15.2.7. Lipolytic Enzymes in Milk Product Manufacture

Most lactic starters used in the manufacture of fermented milk products have weak lipolytic activity due to intracellular lipases and esterases (Fryer *et al.*, 1967; Oterholm *et al.*, 1968; Paulsen *et al.*, 1980). The enzymes, present in the cytoplasm, are released in cheese as the starter cells lyse during maturation. In general, the lipases have pH and temperature optima around 6–7 and 37°C, respectively. They have specificity for short-chain fatty acids and show a preference for partial glycerides over triglycerides (Stadhouders and Veringa, 1973; El Soda *et al.*, 1986). The main lipolytic action of starter bacteria in cheese may be to hydrolyze further monoglycerides and diglycerides produced by other lipases. Lipases of adventitious organisms such as yeasts, lactobacilli, and micrococci may also contribute to lipolysis during cheese ripening (Chapman and Sharpe, 1981; Gripon *et al.*, 1991).

*Penicillium roqueforti* and *P. camemberti* produce very active extracellular lipases, which are the principal lipolytic agents in mold-ripened cheeses. They preferentially hydrolyze the short-chain fatty acids in milk fat. *P. roqueforti* produces two lipases, one with an alkaline pH optimum and the other most active at pH 6–6.5, with slightly differing fatty acid specificities (Menassa and Lamberet, 1982). *P. camemberti* secretes a single lipase with optimal activity at pH 9 (Lamberet and Lenoir, 1976).

Lipase preparations from numerous microorganisms, including those mentioned above, have been used in the synthesis of “dairy” (buttery or cheesy) flavors from milk fat (Arnold *et al.*, 1975; Kilara, 1985) or to enhance flavor development in ripening cheese (Fox, 1988). These include lipases from *Rhizomucor* (*Mucor*) *miehei* (Moskowitz *et al.*, 1977; Hüge-Jensen *et al.*, 1987), *Achromobacter lipolyticum* (Khan *et al.*, 1967), *Aspergillus niger*

(Fukumoto *et al.*, 1963), *A. oryzae* (Arbige *et al.*, 1986), *Geotrichum candidum* (Jensen, 1974; Baillargeon and McCarthy, 1991), *Candida cylindracea* (Benzonana and Esposito, 1971), *C. lipolytica* (Alifax, 1979), *Rhizopus delemar* (Fukumoto *et al.*, 1964), and *Rh. arrhizus* (Verhaeghe *et al.*, 1990). The diversity of properties of these lipases, such as pH optimum and, in particular, specificity, enables the selection of an appropriate enzyme for a specific purpose (Kilara, 1985; Fox and Grufferty, 1991).

One such purpose is to modify milk fat to improve its nutritional properties using a lipase with *sn*-1,3 specificity and fatty acid specificity to reduce its level of long-chain saturated fatty acids (Patel and Thakar, 1994). This has been achieved by using a lipase immobilized onto hydrophobic hollow fibres and carrying out the hydrolysis/interesterification reaction in a solvent-free system under controlled water activity (Balcao *et al.*, 1998). In another report, Garcia *et al.* (1998) used immobilized microbial lipases to enrich milk fat with conjugated linoleic acid, an anticarcinogenic fatty acid naturally present in low amounts in milk fat (Parodi, 1994; see Chapters 3 and 13). Safari and Kermasha (1994) used four commercial lipases to alter the positional structure of milk fat and found that three of the lipases enriched the triacylglycerols with palmitic acid at the *sn*-2 position, an important attribute of human milk fat (Innis *et al.*, 1994).

Pregastric esterases are used in the manufacture of Italian cheeses to produce the characteristic “piccante” flavors (Fox and Guinee, 1987; Birschbach, 1992). These flavors are due to short-chain fatty acids, especially butyric, which are released preferentially from milk fat by these enzymes (Nelson *et al.*, 1977). Pregastric esterases are produced by the salivary glands and can be obtained from the abomasum of milk-fed calves, lambs and kids. They have been isolated in heterogeneous form and have a molecular weight of approximately 172 000 (calf), 168 000 (kid), and 150 000 (lamb) Da (Lee *et al.*, 1980). They are optimally active at 32–42°C and pH 4.8–5.5 (Richardson and Nelson, 1967). Pregastric esterases also find use in cheese flavor development and in flavor ingredient manufacture (Kilara, 1985; Fox, 1988).

Picon *et al.* (1997) included phospholipase C with encapsulated proteinases added to milk to stimulate the release of proteinase in Manchego cheese. The phospholipase, acted on the soy lecithin used to form the capsules, accelerated ripening of the cheese.

### 15.3. Causes of Hydrolytic Rancidity in Milk and Milk Products

Raw cows' milk contains a relatively large amount of lipase activity, but seldom undergoes sufficient lipolysis to cause an off-flavor. Under optimal conditions, the lipase (milk LPL) can catalyze the hydrolysis of up to *ca.*

2  $\mu\text{mol}$  of triglyceride/ml/min (Egelrud and Olivecrona, 1972). Since milk with *ca.* 2  $\mu\text{mol}$  FFA/ml has a rancid flavor (Lombard and Bester, 1979), it is of interest to ascertain why excessive lipolysis occurs in milk only under certain conditions.

Milk when freshly secreted from a healthy udder has  $\leq 0.5$   $\mu\text{mol}$  FFA/ml (Connolly *et al.*, 1979; Bråthen, 1980). These acids result from incomplete synthesis rather than lipolysis. Under proper handling and storage conditions, only small increases in the FFA level should occur. In some cases, however, substantial increases are observed, which result from either “induced” or “spontaneous” lipolysis. “Induced” lipolysis results when the milk lipase system is activated by physical or chemical means. “Spontaneous” lipolysis is defined as that which occurs in milk which has had no treatment other than cooling soon after milking (Tarassuk and Frankel, 1957).

Mastitis and microbial contamination can also contribute to hydrolytic rancidity. In general, lipolysis caused by indigenous milk lipase accounts for most of the rancidity in raw milk and cream; microbial lipolysis is of minor practical importance as little if any lipolysis occurs before the bacterial population reaches  $10^6$ – $10^7$  cfu/ml (Suhren and Reichmuth, 1990). However, in stored milk products, lipolysis by microbial lipases is of greatest significance. Short shelf-life products such as pasteurized milks may be affected by pre-pasteurization lipolysis caused by milk lipase but may be affected by bacterial lipolysis at the end of their shelf-life (Deeth *et al.*, 2002).

Lipolysis in milk is affected by inhibiting and activating factors. As discussed above, proteose peptone fraction of milk can inhibit milk LPL while apolipoproteins stimulate the enzyme. This is particularly important in spontaneous lipolysis; however, proteose peptone 3 has been shown to inhibit lipolysis induced by homogenization, sonication, and temperature activation (Arora and Joshi, 1994), while protein components of the milk fat globule membrane inhibit lipolysis caused by bacterial lipase (Danthine *et al.*, 2000). Several exogenous chemical agents can also inhibit lipolysis (Collomb and Spahni, 1995). For example, polysaccharides such as  $\lambda$ -carrageenan at  $\sim 0.3$  g/l effectively inhibits lipolysis in milk activated by mechanical means or temperature manipulation (Shipe *et al.*, 1982) and lipolysis caused by the lipase from *P. fluorescens* (Stern *et al.*, 1988).

### 15.3.1 Induced Lipolysis

#### 15.3.1.1. Agitation and foaming

Lipolysis in raw milk can be readily initiated by vigorous agitation, causing foaming. Such treatment disrupts the milk fat globule membrane and renders the milk triglycerides more accessible to milk lipase. Incorporation of air (or other gas) and consequent foam formation are essential for damage

to the milk fat globule and for the activation of lipolysis (Tarassuk and Frankel, 1955). The damage results from the high interfacial (liquid-air) tension acting on a small region of the fat globule surface (Mulder and Walstra, 1974).

The amount of lipolysis induced depends on the mode of agitation (e.g., air agitation, pumping, stirring), the severity and duration of agitation, the amount of lipase present, the content and hardness of the fat, and the vulnerability of the milk fat globule membrane (Dunkley and Smith, 1951; Claypool, 1965; Henningson and Adams, 1967; Deeth and Fitz-Gerald, 1977). Milk from cows in late lactation and milk with a tendency to spontaneous lipolysis are quite susceptible to agitation-induced lipolysis (Whittlestone and Lascelles, 1962; Ortiz *et al.*, 1970).

The temperature of the milk during agitation has a major influence. In general, activation is greatest at 37–40°C and least at cold storage temperatures (<5°C). However, the relationship between temperature and activation is complex, and depends on the conditions of the mechanical treatment, the characteristics of the milk, and its age and previous thermal history (Deeth and Fitz-Gerald, 1977). Under certain conditions, the amount of induced lipolysis shows maxima at 12–15°C and 30–40°C with a minimum at 20°C (Fitz-Gerald, 1974; Deeth and Fitz-Gerald, 1977; Hisserich and Reuter, 1984). Hisserich and Reuter (1984) found that, at each temperature, there is a threshold mechanical stress intensity above which lipolysis is induced.

The level of agitation-induced lipolysis depends on the nature and extent of fat globule damage. The extent of damage has been estimated by the amount of free fat in the milk (Te Whaiti and Fryer, 1976), the amount of fat in the skim milk (Hlynka *et al.*, 1945; Aule and Worstorff, 1975), and by the amount of milk fat globule-associated enzymes (e.g., alkaline phosphatase or Xanthine oxidoreductase) released from the fat globules (Stanard, 1975). While good correlations are observed between these parameters and the amount of induced lipolysis for agitation at a given temperature, poor correlations are found for treatments at different temperatures. This can be explained in terms of the agitation causing either aggregation or dispersion of the milk fat globules, with the predominant effect being determined by the temperature of the milk, and the severity of agitation (Deeth and Fitz-Gerald, 1978). A better indication of fat accessibility can be obtained from the amount of lipolysis, which results on addition of a lipase that does not attack the fat of intact milk globules, [e.g., that of *Candida cylindracea* (Deeth and Fitz-Gerald, 1978)].

Besides its effect on the integrity of the milk fat globules, agitation redistributes lipase between the skim milk and cream phases (Frankel and Tarassuk, 1959). Agitation of milk at 5–10°C or 37°C results in a severalfold

increase in lipase activity associated with the cream (Deeth and Fitzgerald, 1977). The amount of lipase transferred to the cream is not reflected in the extent of lipolysis in whole milk, but it is in the lipolysis in cream separated from activated milk. Since the transferred enzyme is bound to the milk fat globule membrane and in this form has enhanced heat stability (Frankel and Tarassuk, 1959), the amount of redistribution is of particular relevance in butter manufacture.

Milking machines are a major cause of on-farm lipolysis (Fleming, 1979; O'Brien *et al.*, 1998; Evers and Palfreyman, 2001). Agitation-induced activation results from faulty design and installation, and inadequate maintenance of milking machines, and is associated with excessive air intake into the system, causing turbulence and frothing (Whittlestone and Lascelles, 1962; Bakke *et al.*, 1983). Features such as elbows, joints, in-line fittings, long and narrow pipes, and vertical risers in the milking line cause turbulence (Kelley and Dunkley, 1954; MacLeod *et al.*, 1957; Speer *et al.*, 1958). High-line milking machines and single-line systems in which milk and air mix intimately are of particular concern (Downes *et al.*, 1974; Rasmussen *et al.*, 1988). In a survey of the FFA level in New Zealand farm milk supplies, Evers and Palfreyman (2001) found that milking machine design was the most important factor, as milk with the lowest level of FFAs was obtained from machines in which the milk and air were transported separately. Heuchel (1994) evaluated 10 types of milking machine clusters and found that a significantly higher level of FFAs resulted from two clusters, which were designed with a cyclic air inlet near the base of the teat. An investigation in Ireland showed that a low claw air admission of 6 l/min and float valves in recorder jars were important factors for controlling lipolysis in milk obtained *via* the mid-level recorder system (O'Brien *et al.*, 1998). Bulk farm milk tanks are rarely implicated in the activation of milk lipase, although excessive agitation has been reported to increase lipolysis (Hunter, 1966). Similarly, road tankers have been found to cause little activation (Kitchen and Cranston, 1969). However, in factory silos, air agitation of milk can cause activation if the air flow-rate is excessive or if agitation is continuous rather than intermittent (Sjöström, 1959).

Continuous pumping, particularly with aeration, causes damage to the milk fat globule membrane and subsequent lipolysis to an extent dependent on the type of pump (Downes *et al.*, 1974; Kirst, 1980). Pumping of raw milk through ultrafiltration membranes (Hicks *et al.*, 1990), and sudden release of pressure and the use of a milk exit temperature of  $>7^{\circ}\text{C}$  during concentration of raw milk by reverse osmosis can also induce lipolysis (de Boer and Nooy, 1980; Barbano *et al.*, 1983). Factory separation of cream, where the cream is partially homogenized as it leaves the separator bowl at relatively

high pressure, can also promote lipolysis (Downes *et al.*, 1974; Anderson *et al.*, 1984).

The extent of lipolysis in raw milk or cream following activation is determined by the temperature and duration of storage. Rapid cooling to, and storage at, a low temperature (without freezing) minimizes lipolysis. The rate of lipolysis falls off with time but can be accelerated by further activation treatments (Downey, 1980).

#### 15.3.1.2. Homogenization

Homogenization of raw milk or cream results in the very strong activation of lipolysis. Milk may become perceptibly rancid within 5 min of treatment (Mulder and Walstra, 1974). Homogenization produces a large surface area of vulnerable milk fat and permits ready access for milk LPL. The activity of the enzyme *per se* is not increased by homogenization. Lipolysis in homogenised milk is related to the pressure, time and temperature of homogenization, [i.e., to the efficiency of milk fat dispersal (Parry *et al.*, 1966)]. The rate of lipolysis in raw milk is greatest immediately after homogenization, then levels off. A second or third homogenization again promotes rapid lipolysis. The slowing and revival of lipolysis is attributable to the accumulation and dissipation of lipolysis products at the interface (Nilsson and Willart, 1960; Downey, 1980).

Lipolysis proceeds readily when pasteurised homogenised milk is mixed with raw, unhomogenised milk. Here, the amount of lipolysis depends on the ratio of the susceptible substrate to the amount of lipase, and is maximal for an approximately 50/50 mixture of the two milks (Nilsson and Willart, 1960). This phenomenon is particularly important in the dairy factory since any recirculation of pasteurised homogenised milk back into the raw milk during start-up or closedown of processing plants can cause appreciable lipolysis. In commercial practice, the homogeniser is placed immediately before or directly after the pasteurizer so that the milk lipase is heat-inactivated before it can cause lipolysis in the homogenised milk.

#### 15.3.1.3. Temperature Activation

Lipolysis may be induced when fresh milk or cream is subjected to a specific sequence of temperature changes. The optimum amount of “temperature activation” is promoted by cooling to  $\leq 5^{\circ}\text{C}$ , warming to  $25\text{--}35^{\circ}\text{C}$ , followed by re-cooling to  $<10^{\circ}\text{C}$  (Krukovsky and Herrington, 1939; Kon and Saito, 1997). Lipolysis proceeds on storage at this low temperature. Re-warming reverses the activation. Milk samples from individual cows vary widely in their susceptibility to temperature activation, the milk from some cows, and even some herds being completely resistant to it. The reasons for

the variability are not clear, although milk susceptible to spontaneous lipolysis is more likely to be affected (Claypool, 1965; Saito and Kim, 1995). The feeding regime of cows may also affect the susceptibility of their milk to “heat-induced” rancidity (Astrup, 1984).

Susceptibility is a property of the cream phase, as has been demonstrated by interchanging cream and skim milk from susceptible and non-susceptible milks (Claypool, 1965; Wang and Randolph, 1978). Kon and Saito (1997) demonstrated that the presence of the MFGM is essential for the phenomenon and suggested that temperature manipulation reduces the protective effect of the membrane and allows the lipase access to the fat. Pasteurised cream can also be activated, and undergoes lipolysis on subsequent mixing with raw (cold) skim milk. The activation treatment facilitates the attachment of the lipase to the surface of the milk fat globules. In contrast to the lipase–milk fat globule membrane interactions that occur in agitation-induced or spontaneous lipolysis, this attachment is reversed by rewarming, but can re-form if the milk is cooled again (Krukovsky and Herrington, 1939; Claypool, 1965).

In practice, temperature activation can occur if a small amount of cooled milk or cream is mixed with a larger amount of warm milk and then re-cooled (McDowell, 1969; Nielsen, 1978). Separation of previously cooled milk at a temperature around 30°C can lead to lipolysis if the cream is held in cold storage before pasteurization.

#### 15.3.1.4. Freezing

Freezing and thawing of milk that leads to churning of the fat (Mulder and Walstra, 1974) may induce lipolysis (Willart and Sjöström, 1966). Slow freezing and repeated freeze–thawing are most effective, but the extent of lipolysis which ensues is less than that promoted by moderate agitation. Freezing of milk in farm bulk tanks is the most likely cause of this activation. A long period of storage, however, can reduce the susceptibility of milk to lipolysis because of the reduction of lipase activity (Needs, 1992).

### 15.3.2. Spontaneous Lipolysis

#### 15.3.2.1. Characteristics

Milk that undergoes lipolysis without being subjected to any of the treatments described above has been referred to as “naturally active,” “susceptible,” “spontaneously lipolytic” or “spontaneous” in contrast to “normal” milk in which no lipolysis occurs.

Spontaneous milk can be produced by most, if not all, cows but, because of the individuality of cows and their response to various factors,

only a proportion of cows in a herd produce such milk at any one time. Reported proportions are between 3 and 35% (Downey, 1980). Spontaneous lipolysis is defined here as that which occurs in some individual milks when cooled to < about 15°C soon after milking.

The sooner spontaneous milk is cooled and the lower the temperature to which it is cooled, the more lipolysis that occurs (Tarassuk and Richardson, 1941; Bachman and Wilcox, 1990a); if cooling is delayed, the extent of lipolysis is reduced (Dunkley, 1946; Kitchen and Cranston, 1969). Once the milk is cooled, spontaneous lipolysis proceeds during cold storage and the rate of lipolysis increases if the temperature is raised (Tarassuk and Richardson, 1941). As with induced lipolysis, the rate of spontaneous lipolysis is high initially but levels off later. An FFA level of up to 10 meq/l can be obtained (in extreme cases) after 24 hours storage at 5°C.

A characteristic of spontaneous lipolysis is that it is inhibited by normal milk. Tarassuk and Henderson (1942) reported that mixing normal and spontaneous milk in the ratio of 3:1 before cooling prevented lipolysis. In fact, the ratio required for complete inhibition depends on the properties of both milks and highly susceptible milk requires a high ratio of normal to spontaneous milk to prevent lipolysis (Deeth and Fitz-Gerald, 1975a). Furthermore, admixing of two spontaneous milks can result in more lipolysis than if the two milks are incubated separately. This phenomenon can be explained in terms of the lipase activity in the two milks and the relative levels of inhibitors and activators present (Section 15.3.2.3) (Deeth and Fitz-Gerald, 1975a). Because of the usual predominance of normal milk over spontaneous milk, lipolysis in bulk herd milk is usually low. A high proportion of spontaneous milk, resulting from a coincidence of some of the factors discussed below, can lead to a high FFA level in bulk milk.

#### 15.3.2.2. Factors Affecting Spontaneous Lipolysis

*Stage of lactation.* The variability in propensity to produce spontaneous milk applies to individual cows within a herd, cows in different herds, cows at different stages of lactation and pregnancy, and even to the same cows from day to day and from lactation to lactation (Fredeen *et al.*, 1951). Stage of lactation is one of the most important factors responsible for this variability, with cows in late lactation having the greatest tendency to produce spontaneous milk (Fredeen *et al.*, 1951; Ortiz *et al.*, 1970; Saito, 1983, 1992; Ahrné and Björck, 1985), with the last two months before drying off being critical (Chazal and Chilliard, 1987b). One of the first flavor defects to be linked with lipolysis was known as “bitter milk of late lactation” (Palmer, 1922). However, cows at any stage of lactation can produce spontaneous milk.

Most investigators have found the interval since calving or length of lactation to be more important than the stage of pregnancy, with long lactations being particularly conducive to the production of spontaneous milk (Fredeen *et al.*, 1951). However, some reports have suggested that the stage of gestation or a combination of gestation stage and low milk yield may be more significant than the stage of lactation (Bachmann, 1961; Chazal and Chilliard, 1986; Bachman *et al.*, 1988).

*Feed and nutrition.* Both the quality and quantity of feed influence the tendency of a cow to produce spontaneous milk (Fredeen *et al.*, 1951; Jellema, 1980). The milk of most cows on a low plane of nutrition has an enhanced susceptibility (Gholson *et al.*, 1966; Astrup *et al.*, 1980). The effect of low energy intake is particularly marked when cows are in late lactation (Stobbs *et al.*, 1973; O'Brien *et al.*, 1996) but can also be considerable in early lactation (Dillon *et al.*, 1997). The cow's body condition has not been found to be a reliable indicator of the susceptibility of her milk to spontaneous lipolysis (Ortiz *et al.*, 1970).

Cows fed a dry ration, particularly hay (Tarassuk *et al.*, 1962) and high carbohydrate winter feeds (Chen and Bates, 1962; Kodgev and Rachev, 1970) have generally been found to be more likely to produce susceptible milk than cows fed green pasture (Tarassuk and Regen, 1943; Fredeen *et al.*, 1951; Jensen *et al.*, 1960). This may explain the report of Alkanhal *et al.* (2000) claiming that milk from cows fed a high (70%) level of concentrate exhibited a higher level of spontaneous lipolysis than milk from cows fed a low (40%) level of concentrate. Lipase activity and initial FFA concentration were also higher in raw milk from cows fed high levels of concentrate. However, Chazal and Chilliard (1986) reported higher milk FFA levels for cows at pasture than when housed. Feeding of silage can result in serious problems with spontaneous lipolysis (Johnson and Von Gunten, 1961; Chazal *et al.*, 1987). In changeover feeding trials, lag times of 4–5 days have been observed for corresponding changes in the susceptibility of the milk (Tarassuk, 1940; Stobbs *et al.*, 1973).

In feeding trials with lipid supplements, Astrup *et al.* (1980) observed that palmitic or myristic acid significantly enhanced spontaneous lipolysis but stearic acid and fatty acids with a chain length shorter than myristic acid had no effect. These workers found that feeding rapeseed oil to underfed cows reduced the susceptibility of their milk to lipolysis, while Chazal and Chilliard (1985) reported that supplementation with non-protected lipids, particularly highly unsaturated oils such as rapeseed, increased the level of FFAs in milk. Protected oil supplements have been found to lead to reduced lipolysis in milk (Astrup *et al.*, 1979) or to have little effect on FFA level (Urquhart *et al.*, 1984).

An in-depth review of the effects of feed and nutrition on spontaneous lipolysis was published by Jellema (1980).

*Season.* Several authors have suggested a seasonal variation in the incidence of spontaneous lipolysis, with most indicating a higher incidence in the colder months (Dunkley, 1946; Kodgev and Rachev, 1970; O'Brien *et al.*, 1999) but others the opposite effect (Hunter *et al.*, 1968; Chazal and Chilliard, 1986). In New Zealand, Evers and Palfreyman (2001) found significantly lower FFA levels in spring milk than in autumn milk. However, it appears that the season *per se* is not the determining factor but rather the stage of lactation/pregnancy of the majority of the cows and the type and availability of feed (Speer *et al.*, 1958; Hunter *et al.*, 1968; Menger, 1975; Chazal and Chilliard, 1986).

*Milk production.* In general, low-yielding cows are more likely to produce spontaneous milk than are high-yielding animals (Hunter *et al.*, 1968; Ortiz *et al.*, 1970; Jellema and Schipper, 1975; Chazal and Chilliard, 1986; Saito, 1992). The milk yield, like season, cannot be considered an independent variable as it is dependent on other factors such as stage of lactation and quality and quantity of feed (Ahrné and Björck, 1985).

There have been several reports that milk obtained at the evening milking is more susceptible to spontaneous lipolysis than that obtained in the morning (Doody *et al.*, 1975; Jellema and Schipper, 1975; Saito, 1983; Ahrné and Björck, 1985). This is attributed to the shorter inter-milking interval before the evening milking, which results in lower milk production (Suhren *et al.*, 1981; Saito, 1983). O'Brien *et al.* (1998) examined the effect of milking interval on the FFA level in the milk from cows with an average daily yield of 25 kg and found no difference between a 16:8 h and a 12:12 h interval.

*Other factors.* A cow's hormonal balance can affect the susceptibility of her milk to spontaneous lipolysis (Fredeen *et al.*, 1951; Kästli *et al.*, 1967; Bachman *et al.*, 1988). The oestrus cycle appears to have little effect on spontaneous lipolysis (Fredeen *et al.*, 1951) but may affect lipase activity in the milk (Kelly, 1945). In contrast, treatment of cows with oestradiol and progesterone has been shown to lead to increased lipolysis in the milk (Bachman, 1982; Heo, 1983; Bachmann *et al.*, 1985) but no change (Bachman, 1982) or a transient increase (Bachmann *et al.*, 1985) in total lipase activity. It appears that the increased lipolysis in milk following hormonal treatment, or in milk from cows with ovarian cysts, may not be typical spontaneous lipolysis as cooling is not needed to initiate it (Bachman, 1982); a lipase other than lipoprotein lipase, possibly a bile salt-stimulated lipase, may be responsible for such lipolysis (Heo, 1983; Bachmann *et al.*, 1985). Treatment of cows with bovine somatotropin has been reported to have no significant effect on milk lipoprotein lipase activity (Azzara *et al.*, 1987).

Research in the Netherlands has indicated that the type of milking machine used can affect the physiology of the cow's udder and thereby influence the susceptibility of the milk to lipolysis (Jellema, 1975). Cows milked with a one-line system produced milk that was more susceptible than milk from the same cows when milked with a two-line system. Shorter times between milking can have a marked effect on the level of spontaneous lipolysis; the milk of some cows changes from normal to spontaneous when milking interval was reduced to 3–4 h (Jellema, 1986). This may be due to the higher LPL activity in milk drawn after a short interval, as the lipase loses activity at body temperature (Olivecrona and Bengtsson-Olivecrona, 1991).

The breed of the cow, generally, does not appear to affect its propensity to produce spontaneous milk (Chilliard, 1982). For example, Chazal and Chilliard (1987a) found no difference between Friesian and Montbéliarde cows in relation to spontaneous lipolysis. Bachman *et al.* (1988), however, found that the milk of Jerseys is more susceptible than that of Holsteins. There also appears to be some within-breed heritability of spontaneous milk production (Deeth and Fitz-Gerald, 1976; Jurczak, 1996).

Bachman *et al.* (1988) reported a low repeatability (0.22) of spontaneous lipolysis in the milk of cows from lactation to lactation. However, according to Chazal and Chilliard (1987c), the repeatability between two successive lactations explained 30–40% of the variation in FFA data. These authors concluded that spontaneous lipolysis evident in late pregnancy is dependent on an intrinsic factor repeatable from lactation to lactation as well as an extrinsic factor, probably linked to diet.

Supplementation of cows' diets with zinc has been found to reduce spontaneous lipolysis significantly (Hermansen *et al.*, 1995). The authors suggested that zinc deficiency may be a potential risk factor for spontaneous lipolysis. However, corroborative evidence is required before zinc supplementation could be recommended for reducing spontaneous lipolysis.

### 15.3.2.3. Biochemical Aspects

*A priori*, one might expect the extent of spontaneous lipolysis in a milk is determined largely by the amount of enzyme present. All milks have sufficient lipase activity to cause extensive lipolysis (Downey, 1980) but this occurs only when other conditions, as outlined below, are conducive to such lipolysis. With few exceptions (e.g., Hemingway *et al.*, 1970), low correlations between lipase activity and the level of spontaneous lipolysis have been reported (Castberg and Solberg, 1974; Driessen and Stadhouders, 1974; Clegg, 1981; Ahrné and Björck, 1985; Cartier and Chilliard, 1990). However, a universal finding is that there is a high correlation between the

amount of lipase bound to the milk fat globule and the degree of lipolysis (Ahrné and Björck, 1985; Sundheim and Bengtsson-Olivecrona, 1985). Conditions that favor dissociation of the lipase from the casein micelles or attachment of the enzyme to the milk fat globule membrane increase the susceptibility of milk to spontaneous lipolysis (Downey and Andrews, 1966; Deeth and Fitz-Gerald, 1975a; Anderson, 1982a; Sundheim and Bengtsson-Olivecrona, 1985).

The susceptibility of the milk fat globule and the permeability of the milk fat globule membrane have been considered to be important in spontaneous lipolysis (Dunkley and Smith, 1951; Bachmann, 1961; Deeth and Fitz-Gerald, 1975a). However, investigations involving intermixing of skim milk and cream fractions from normal and spontaneous milks have indicated that the extent of lipolysis is dependent more on the skim than on the cream portion (Claypool, 1965; Deeth and Fitz-Gerald 1975a; Murphy *et al.*, 1979). Cartier and Chilliard (1990) obtained a correlation coefficient of +0.57 between spontaneous lipolysis and the susceptibility of the fat to *Candida cylindracea* lipase, a measure of the condition of the milk fat globule membrane (Deeth and Fitz-Gerald, 1978). However, Sundheim and Bengtsson-Olivecrona (1987a) observed only slight differences in the behavior of fat globules isolated from normal or spontaneous milks as substrates for purified lipoprotein lipase. One difference observed was the shorter lag time for spontaneous fat globules before lipolysis commenced. The state of the milk fat globule membrane may be most significant as a factor in spontaneous lipolysis in late lactation milk (Bachmann, 1961; Deeth and Fitz-Gerald, 1975a) where the amount of phospholipid may not be enough for the formation of sufficient membrane to cover the increased surface area (at least twice that of mid-lactation milk) of the fat globules (Kinsella and Houghton, 1975). A further indication of the importance of the condition of the milk fat globule membrane is the greater susceptibility of spontaneous and late-lactation milks to induced lipolysis (Whittlestone and Lascelles, 1962; Jellema and Schipper, 1975).

In spontaneous lipolysis, the milk lipase attaches to the milk fat globule membrane during cooling (Ahrné and Björck, 1985; Hohe *et al.*, 1985; Sundheim and Bengtsson-Olivecrona, 1985). This interaction does not occur if the milk is not cooled, is reduced if cooling is delayed, but is not disrupted if milk is rewarmed after being cooled (Sundheim and Bengtsson-Olivecrona, 1987a). The formation of this enzyme-membrane complex appears to be an essential feature of spontaneous lipolysis as no lipolysis occurs if the interaction is prevented by delayed cooling, addition of NaCl or other inhibitory factors (see below) (Deeth and Fitz-Gerald, 1975a; Sundheim and Bengtsson-Olivecrona, 1987a). The role of the milk fat globule membrane may be a rather complex regulatory one since it contains both activating

lipoproteins (Castberg and Solberg, 1974) and inhibitory proteins (Shimizu *et al.*, 1982; Sundheim and Bengtsson-Olivecrona, 1987b). Furthermore, since milk fat globules from both normal or spontaneous milk become receptive to attachment of lipoprotein lipase (and heparin) when cooled in the absence of skim milk, the membrane must undergo some conformational change such that its affinity for lipase (and heparin) increases (Sundheim and Bengtsson-Olivecrona, 1987d).

Following the discovery that the major lipolytic enzyme in milk is a lipoprotein lipase and that lipolysis could be initiated in any milk by the addition of blood serum or serum lipoproteins (Jellema, 1975), it was suggested that spontaneous milk contains activating cofactors derived from the blood (Jellema and Stadhouders, 1974). Some considered that these cofactors may be small (molecular weight *ca.* 10 000 Da) soluble apo-LPs, which could leak into the milk from the blood during times of physiological stress (Olivecrona, 1980). The blood lipoproteins are much larger and are less likely to be transferred to the milk. However, the work of Clegg (1980) and Driessen and Stadhouders (1974) indicate that the lipid component is essential for activating lipolysis. Milk has been shown to contain material that is immunologically cross-reactive with bovine serum lipoproteins (Castberg and Solberg, 1974; Anderson, 1979). Bachman and Wilcox (1990b) found that added HDL, the major lipoprotein responsible for activation of lipolysis (Sundheim *et al.*, 1983) redistributes the lipoprotein lipase towards the milk fat globules rather than activating already-bound enzyme, and suggested that HDL in milk could be responsible for spontaneous lipolysis.

The presence of an inhibitory factor (or factors) in milk has been suggested to explain the lack of lipolysis in normal milk and the inhibition of lipolysis when normal milk is mixed with spontaneous milk (Dunkley and Smith, 1951). It has been demonstrated that normal skim milk contains a heat-stable, dialysable inhibitor (Deeth and Fitz-Gerald, 1975a), and that proteose-peptone 3 is an effective non-competitive inhibitor (Anderson, 1981; Cartier *et al.*, 1990). The inhibitors prevent lipolysis by blocking the lipase–milk fat globule membrane interaction (Deeth and Fitz-Gerald, 1975a).

The activator–inhibitor balance in the skim fraction of milk appears to be a major determinant of the extent of spontaneous lipolysis (Deeth and Fitz-Gerald, 1975a; Sundheim and Bengtsson-Olivecrona, 1987c; Cartier and Chilliard, 1990). Thus, depending on the relative amounts of activators and inhibitors, skim milk or milk serum can act as an inhibitor or an activator, but are most commonly inhibitory. The inhibiting/activating nature of a skim milk can be assessed by preincubating it, after heating to destroy indigenous LPL, with a substrate emulsion such as Intralipid and

then determining the resulting lipolysis on incubation with purified LPL (Cartier and Chilliard, 1990), or by observing the effect of heated skim milk on the interaction of purified lipoprotein lipase with washed, pooled milk fat globules during cooling, and the subsequent amount of lipolysis (Sundheim and Bengtsson-Olivecrona, 1987c).

Thus, four factors have been shown to contribute to the susceptibility of a milk to spontaneous lipolysis: lipase activity, milk fat globule vulnerability, activating factors and inhibiting factors, with the balance of the last two being most important (Deeth and Fitz-Gerald, 1975a; Sundheim, 1988; Cartier and Chilliard 1990). Sundheim (1988) concluded that these factors could explain 80–87% of lipolysis induced by cold storage.

### 15.3.3. Mastitis

Mastitis has often been considered to be a cause of spontaneous lipolysis (Downey, 1980) because many mastitic milks have an elevated level of FFAs (Tarassuk and Yaguchi, 1958; Tallamy and Randolph, 1969; Fitz-Gerald *et al.*, 1981). These levels tend to increase with increasing somatic cell count (SCC). However, many milks that can be classified as mastitic (SCC  $>0.5 \times 10^6$ /ml; Kästli, 1967) do not have an elevated level of FFAs relative to corresponding healthy quarters; the percentage of milks exhibiting lipolysis increases approximately linearly up to 100% at SCC of  $\geq 10 \times 10^6$ /ml (Fitz-Gerald *et al.*, 1981).

The data of Gudding (1982) suggest that the elevation of FFAs may depend on the cause of mastitis, as relatively higher levels of FFAs were observed in milk from quarters infected with *Staphylococcus aureus*. When mastitis is induced experimentally by intramammary infusion of endotoxins or bacteria, the increases in FFAs correspond closely with the increases in SCC and other indices of mastitis (Salih and Anderson, 1979; Fitz-Gerald *et al.*, 1981; Ma *et al.*, 2000). Murphy *et al.* (1989) concluded that the increased lipolysis in mastitic milk is due to increased susceptibility of the milk fat.

Mastitic milk samples have higher initial levels of FFAs than normal milk (Tarassuk and Yaguchi, 1958; Fitz-Gerald *et al.*, 1981; Murphy *et al.*, 1989; Van den Heever *et al.*, 1990). They also show greater increases in FFAs during storage than milk from healthy quarters (Fitz-Gerald *et al.*, 1981; Van den Heever *et al.*, 1990) but these increases are small compared with those observed in spontaneous milks. For these reasons, mastitis is not considered to be a cause of spontaneous lipolysis as defined here. The relationship between the level of milk lipase and mastitis has been the topic of numerous investigations. Some surveys have shown reduced milk

lipase activity in mastitic milk (Fitz-Gerald *et al.*, 1981; Murphy *et al.*, 1989), an effect attributed to impaired biosynthetic capability of the mammary tissues caused by infection and inflammation or by the action of intracellular proteinases from somatic cells on the milk lipase (Jurczak and Sciubisz, 1982). Other reports have indicated a higher level of lipase in mastitic milk (Tallamy and Randolph, 1969; Azzara and Dimick, 1985b). The lipase in mastitic milk has a different distribution from that of normal milk, with a higher proportion being in non-micellar form, either soluble (Salih and Anderson, 1979) or associated with the cream (Bachmann, 1961).

The leucocytes in milk contain a lipase (Gaffney and Harper, 1965; Azzara and Dimick, 1985a) or carboxylesterase (Deeth, 1978), which may contribute to lipolysis in mastitic milk. When suspensions of these cells are added to milk, the level of FFAs increases, almost linearly up to a cell count of *ca.*  $2 \times 10^6$ /ml (Salih and Anderson, 1978). More lipolysis is observed if the cells are disrupted prior to addition to milk (Jurczak and Sciubisz, 1981).

Azzara and Dimick (1985a) reported that  $2.5 \times 10^6$  macrophages/ml would contribute 1% of total lipase activity on addition to milk, but this value increased to 11.6% after 48 h storage. They suggested that the macrophage lipases may bind to the milk fat globule membrane and activate the substrate to the action of milk lipase.

#### 15.3.4. Microbial Lipolysis

Hydrolytic rancidity can occur in milk and milk products as a result of contamination by microorganisms premanufacture or postmanufacture (Lawrence, 1967). The introduction of bulk cold storage has led to the emergence of psychrotrophic (mainly Gram-negative) bacteria as the dominant organisms in raw milk and cream (Thomas and Thomas, 1978; Cousin, 1982). Storage of milk at the farm and factory can extend its age at processing to several days, and allow sufficient growth for spoilage, including lipolysis, to occur. Only a proportion of psychrotrophs (between 0.1 and *ca.* 30%, depending on the composition of the flora of the sample (Chapman *et al.*, 1976; Muir *et al.*, 1978a) produces appreciable amounts of lipolytic enzymes, so that there is not necessarily a good correlation between psychrotroph numbers and FFA or rancid flavor (Muir *et al.*, 1978b). Muir *et al.* (1978a) observed lipolysis in stored farm or factory milks only when psychrotroph counts were  $> 5 \times 10^6$ /ml.

Lipolysis can occur in pasteurised milk as a result of post-pasteurization contamination by lipolytic psychrotrophs. In whole milk, this is a major cause of spoilage while in skim milk, proteolysis is the major cause

(Deeth *et al.*, 2002). In a study of the bacteriological quality of pasteurized milks from three Australian factories, Craven and Macauley (1992) concluded that the standard of hygiene in the factory (as indicated by the number of contaminants in the milk immediately after processing) and the lipolytic activity of the stains that grow to a sufficient number to cause spoilage are the most important factors determining the keeping quality of the milk.

Lipolysed off-flavor is one of the most common flavor defects in (pasteurised) market cream (Mottar, 1989; Jensen and Poulsen, 1992). Development of flavor defects in pasteurized milk and cream is associated with contamination by high numbers (e.g.,  $> 10^7$ /ml) of psychrotrophs (Bandler *et al.*, 1981). Taints may be evident after 4–5 days at 5°C (Hawney and Royal, 1970) or after a shorter time at a higher temperature (Muir *et al.*, 1978a). In cultures of single lipolytic psychrotrophs, a level of approximately  $10^6$ /ml (Suhren *et al.*, 1977) or  $10^7$ /ml (Overcast and Slean, 1959; Shelley *et al.*, 1986) is attained before spoilage is apparent. Psychrotrophic spore-formers can cause lipolytic spoilage in milk heat-treated at greater than 72°C  $\times$  15 s but less than UHT (Eibel and Kessler, 1987).

Heat-stable bacterial lipases have been implicated in the development of rancidity during storage of products, [e.g., UHT milk (Mottar, 1981), butter (Nashif and Nelson, 1953; Deeth *et al.*, 1979), and cheese: Cheddar (Law *et al.*, 1976; Cousin and Marth, 1977), Dutch (Driessen and Stadhouders, 1971), Swiss (Pinheiro *et al.*, 1965), Teleme (Kalogridou-Vassiliadou and Alichanidis, 1984) and Camembert (Dumant *et al.*, 1977)] made from milk containing a high number of psychrotrophs (usually  $> 10^7$ /ml) before UHT or HTST treatment. However, Adams and Brawley (1981) reported that UHT milk with counts of  $10^4$ – $10^5$  cfu/ml before processing lipolysed on storage at 25 or 40°C. Celestino *et al.* (1996) reported that in UHT milk produced from reconstituted powder made from cold-stored (48 h at  $\sim 4^\circ\text{C}$ ) raw milk, bacterial lipases (and proteinases) were reactivated during storage and that the taste of reconstituted UHT milk was affected more by lipolysis than by proteolysis.

The lipases have been found to be concentrated in the cream on separation and in the curd on rennet-induced coagulation (Kishonti and Sjöström, 1970; Driessen and Stadhouders, 1975; Stead, 1983). They can survive during the manufacture of dried milk (Shamsuzzaman *et al.*, 1987) and cause lipolytic defects in a wide range of fat-containing foods in which milk powder is an ingredient (Stead, 1986).

Psychrotrophic yeasts, molds and bacteria can cause rancidity and surface spoilage in butter (Thomas and Druce, 1971), a problem less significant now than previously due to improved factory methods and hygiene.

## 15.4. Detrimental Effects of Lipolysis in Milk and Milk Products

### 15.4.1. Flavor Defects

#### 15.4.1.1. Milk and Cream

The relationship between the flavor of milk and its FFA content has been examined by numerous workers, and threshold FFA levels for the detection of rancid flavor have been established. Many of these studies have been reviewed (Kuzdzal-Savoie, 1975, 1980; Connolly *et al.*, 1979; Anderson *et al.*, 1991).

The threshold value reported shows considerable variation (Table 15.1). The wide range can be attributed largely to variations in the method (Section 15.6.1) used to obtain the thresholds. Furthermore, individual people differ widely in their ability to detect rancid flavor, according to their natural flavor perception and their degree of training in tasting (Anderson, 1983; Bodyfelt *et al.*, 1988; Duncan *et al.*, 1991). This is particularly evident in studies in which taste panellists have been used (e.g., MacLeod *et al.*, 1957; Connolly *et al.*, 1979). Some authors have reported a low correlation between FFA level and rancidity score. Duncan *et al.* (1991) reported a correlation coefficient of 0.27 for laboratory-prepared rancid samples; however, a much better correlation ( $r = 0.93$ ) was found for farm milk samples. For pasteurized milk, Christen *et al.* (1992) reported a significant correlation between levels of FFAs and sensory evaluation by both a trained panel and experts on day 1 but the correlation was lower on day 15. It was suggested that other flavors may have confounded the results for the stored samples.

**Table 15.1.** Level of Free Fatty Acids (FFA) (meq/100 g fat) in Normal and Rancid Milk

Normal value	Typical range	Threshold value for off-flavor	Typical range
0.25–0.4 (Christen, 1993); <1 (Renner <i>et al.</i> , 1989)	0.3–1.0 IDF(1987)	0.7* (Magnusson, 1974); >1.0 (Azzara and Campbell, 1992); >1.2 (Christen, 1993); 1–1.5 (Allen, 1994); 1.5–2 (Downey, 1980); 2.0* (Bråthen, 1980); >2 (Bodyfelt <i>et al.</i> , 1988); 2.0–2.2 Tallamy and Randolph (1969); 2.74 (Kintner and Day, 1965); 1.46–3.62 (Duncan <i>et al.</i> , 1991).	1.5–2.0 IDF (1987)

\* These values are in meq/l

The presence of other flavors may explain the curious situation with UHT milk for which Collins *et al.* (1993) reported a lack of significant correlation between rancidity score and the extent of lipolysis. In other studies, UHT milk samples with an acid degree value (ADV) from 1.2 to 3.0 meq/100 g fat were reported to show no lipolysed flavor (Hansen and Swartzel, 1980; Earley and Hansen, 1982; Rerkrai *et al.*, 1987) while other researchers have shown that when the ADV exceeds 1.5 meq/100 g fat, UHT milk is judged to be rancid (Renner, 1988). One possible explanation is the different patterns of fatty acids released during the storage of raw milk. Choi and Jeon (1993) reported that the fatty acids released were largely long-chain acids which make a low contribution to rancid flavor. Choi *et al.* (1994) subsequently isolated two types of lipase from UHT milk and found that one, associated with the milk fat globules, released predominantly palmitic and stearic acids, while the other from the serum fraction released mostly butyric, followed by caproic and palmitic acids. They suggested that the former activity was due to microbial lipase while the latter was due to milk LPL. The presence of a serum-activated lipase, possibly milk LPL, in UHT milk was also reported by Pande and Mathur (1990, 1992), who found a substantial increase in this activity during storage but no lipolysed flavor was detected during storage. The presence of active milk LPL in UHT milk is unexpected given the heat lability of this enzyme (Section 15.2.1).

The thresholds given by Tallamy and Randolph (1969) (for non-UHT milk) appear to be reasonable and generally acceptable, (i.e., 1.2–1.5 meq/100 g fat for trained experts and 2.0–2.2 meq/100 g fat for the average consumer). A report by the IDF (1987) concluded that rancidity is normally detected between 1.5 and 2.0 meq/100 g fat. Rancidity is detected at approximately the same levels in cream (Dunkley, 1951; IDF, 1991). Similar numerical levels apply to milk where FFA content is measured by an extraction–titration method and expressed as meq/l milk (Bråthen, 1980).

Rancid flavor in milk is not due to a single fatty acid (Bassette *et al.*, 1986; Bodyfelt *et al.*, 1988; Jeon, 1994), but to a mixture of fatty acids, mainly of chain lengths  $C_{4:0}$ – $C_{12:0}$  (Kolar and Mickle, 1963; Al-Shabibi *et al.*, 1964; Scanlan *et al.*, 1965; Allen, 1994; Jeon, 1994), although some bitterness may be due to partial glycerides (Jensen, 1964). The long-chain acids containing 14 to 18 carbons make little contribution to flavor. Al-Shabibi *et al.* (1964) concluded that although the  $C_{4:0}$ – $C_{8:0}$  acids constitute part of the rancid flavor, the  $C_{10:0}$  and  $C_{12:0}$  acids are responsible for most of the unclean, bitter, soapy flavor of lipolysed milk.

The theoretical concentrations of the  $C_{4:0}$ – $C_{12:0}$  fatty acids in milk with a total FFA content of 2 meq/l are shown in Table 15.2, together with the range for rancid milks found by Kintner and Day (1965) and the threshold levels for these individual acids reported by Scanlan *et al.* (1965).

**Table 15.2.** Concentration (mg/kg) of Free C<sub>4:0</sub>–C<sub>12:0</sub> Fatty Acids in Rancid Milk

Fatty acid	Theoretical <sup>a</sup> for milk with 2 meq/l	Found for range of rancid milks <sup>b</sup>	Threshold level of added acids <sup>c</sup>
C <sub>4:0</sub>	26.6	27.5–85.0	46.1
C <sub>6:0</sub>	15.1	16.0–48.7	30.4
C <sub>8:0</sub>	9.1	8.3–27.9	22.5
C <sub>10:0</sub>	17.6	27.6–78.6	28.1
C <sub>12:0</sub>	17.6	26.7–63.3	29.7

<sup>a</sup> Assuming an average molecular weight of 228 Da and the FFA profile reported by Day (1966).

<sup>b</sup> The range of concentrations of FFAs from threshold rancidity to extremely rancid (Kintner and Day, 1965).

<sup>c</sup> Amounts added to fresh, pasteurized, homogenized milk to produce a rancid flavor (Scanlan *et al.*, 1965).

It is clear that the levels of the individual acids in rancid milk can be considerably lower than the threshold values reported by Scanlan *et al.* (1965), which do not include the levels contributed by the fresh milk, (i.e., approximately half the concentrations shown in the second column). Thus, the flavor of the combination of the acids in rancid milk is apparently sufficient to exceed the threshold for detection of rancidity.

In milk with a normal pH of ~6.7, most of the acids are in the salt form and have much less flavor than if they were completely in the acid form (Kuzdzal-Savoie, 1980). In fact, acidification of milk greatly enhances the sensitivity of organoleptic detection of lipolysis in milk (Tuckey and Stadhouders, 1967). The detection of rancidity is reduced by the association of the FFAs with certain proteins in milk (Parks and Allen, 1979; Keenan *et al.*, 1982) and by heating of milk (Kintner and Day, 1965). The phase in which the fatty acids are soluble also influences their flavor threshold, since the short-chain acids have much lower thresholds in fat than in water, while the opposite applies to the long-chain acids (Patton, 1964). For example, butyric acid (C<sub>4:0</sub>) has a flavor threshold of 7 mg/kg in water, but only of 0.6 mg/kg in oil (Delahunty and Piggott, 1995).

#### 15.4.1.2. Butter

Hydrolytic rancidity in butter is characterised by off-flavors variously described as “bitter,” “unclean,” “wintery,” “butyric,” “rancid” or “lipase.” The defect may be evident at manufacture (Störgards and Magnusson, 1966), but is more likely to develop during storage (O’Connell *et al.*, 1975; Bell and Parsons, 1977; Azzara and Campbell, 1992; Champagne *et al.*, 1994).

The concentration of FFAs in butter is usually expressed as ADV in meq/100 g fat (or mg NaOH/100 g fat; 1 meq = 40 mg NaOH). As in the

case of milk, numerous workers have endeavored to correlate FFA level with flavor to establish a threshold for rancidity. In many studies, little or no correlation has been observed between ADV and "lipase" defects (Bell and Parsons, 1977; Connolly *et al.*, 1979). However, Woo and Lindsay (1983a) established a statistical correlation between flavor and individual FFAs. Investigators have reported thresholds ranging from 0.75 to 2.8 meq/100 g fat (Krukovsky and Herrington, 1942a; Downey, 1980; Woo and Lindsay, 1983a). A threshold value of 1.5 meq/100 g fat appears to be a realistic guide for the butter manufacturer (Stadhouders, 1972). Besides lipolytic defects, butter with an ADV  $> 1.5$  meq/100 g fat is likely to have other defects, (e.g., oxidised flavors), and to deteriorate during storage (Deeth *et al.*, 1979). There is some evidence that free fatty acids oxidize more readily than esterified acids (Mukherjee, 1950) and hence may predispose butter to oxidative rancidity (Badings, 1970).

The difficulty in relating rancid flavors in butter to FFA content arises because the short-chain acids, C<sub>4:0</sub> and C<sub>6:0</sub>, which are the most flavorsome (McDaniel *et al.*, 1969), are water-soluble and hence are mostly lost in the buttermilk and wash water during the manufacture of butter. For this reason, even butter made from cream with an ADV as high as 2.4 meq/100g fat may show little defect while on the other hand, butter with quite a low ADV can be rancid, particularly if lipolysis occurs after manufacture (Deeth *et al.*, 1979; Woo and Lindsay, 1980).

The flavor thresholds for the individual fatty acids are quite different in butter and in milk. Whereas in milk, C<sub>10:0</sub> and C<sub>12:0</sub> acids are most significant to rancid flavor, in butter C<sub>4:0</sub> and C<sub>6:0</sub> are of most interest since they have much lower flavor thresholds in fat than do the longer-chain acids (Patton, 1964). The reported thresholds of the C<sub>4:0</sub> to C<sub>12:0</sub> acids added singly or in pairs to butter are shown in Table 15.3, together with the theoretical amounts for an increase in ADV of 0.1 meq/100 g fat. From these data, it is evident that a low level of lipolysis in butter produces sufficient butyric acid to exceed its flavor threshold and to impart a rancid flavor. Thus, measurement of C<sub>4:0</sub>, C<sub>6:0</sub> and C<sub>8:0</sub> gives the best indication of hydrolytic rancidity in butter (McNeill *et al.*, 1986).

The above discussion applies to sweet-cream butter only. Little information is available on cultured butter, but O'Connell *et al.* (1975) found that ripened-cream butter is less prone to the development of hydrolytic rancidity than the corresponding salted or unsalted sweet-cream butter.

#### 15.4.1.3. Cheese

The typical flavor of aged cheese is due to the combination of a variety of flavor compounds, including FFAs (Law, 1984). When excessive lipolysis

**Table 15.3** Concentrations of C<sub>4:0</sub>–C<sub>12:0</sub> Free Fatty Acids (FFA) in Butter

Fatty acid	FFA level (mg/kg)		
	Flavor thresholds <sup>a</sup>		Theoretical <sup>b</sup> increase for ΔADV = 0.1 meq/100 g fat
	Added singly	Added in pairs <sup>c</sup>	
C <sub>4</sub>	11.4	4–7	11.3
C <sub>6</sub>	51.5	16–26	6.4
C <sub>8</sub>	454.6	148–367	3.9
C <sub>10</sub>	161.6	132–173	7.4
C <sub>12</sub>	127.9	70–84	7.4

<sup>a</sup> For acids added to good quality butter (McDaniel *et al.*, 1969).

<sup>b</sup> Based on the FFA profile of rancid milk (Day, 1966) and assuming average molecular weight of 228 Da and 82% fat in butter.

<sup>c</sup> Range of thresholds when FFA was tested in pairs with each of the other 4 FFAs in this table  
ADV = acid degree value.

occurs in cheese, or in the cheesemilk before manufacture, this balance is upset and rancid flavors result (Woo *et al.*, 1984; Fox and Law, 1991). Pasteurised-milk cheeses have lower levels of FFAs than raw-milk cheeses, for example, 38% lower in Manchego (Gaya *et al.*, 1990) and 50% lower in Cheddar (McSweeney *et al.*, 1993). The difference has been attributed to the higher numbers of non-starter lactic acid bacteria (McSweeney *et al.*, 1993) and the indigenous milk lipase (Grappin and Beuvier 1997) present in raw-milk cheese. Vlaemynck (1992) showed that milk LPL may contribute to lipolysis in raw-milk Gouda-type cheese as it retained some activity at pH 5.3 and in 1 M NaCl. Apart from lipolysis due to milk LPL or contaminant microbial lipases, rancid flavors may arise through the addition of lipases or esterases to accelerate flavor development (Law and Wigmore, 1985) or from lipase-contaminated microbial rennet preparations.

Because of the high total flavor of cheese, the threshold levels of FFAs are higher than for milk or butter. In Cheddar cheese, ADVs of 2.8–3.0 meq/100 g fat are usually attained before rancidity is evident (Deeth and Fitzgerald, 1975b). Various studies have shown that rancid Cheddar has 2–10 times more FFAs than good quality cheese (Bills and Day, 1964; Ohren and Tuckey, 1969; Law *et al.*, 1976).

Levels of individual short-chain FFAs or combinations of these have been suggested as superior to total FFA or ADV as indicators of the desirable/undesirable lipolysis status and flavor potential of various cheeses, in particular butyric acid (C<sub>4:0</sub>) and total short-chain FFAs (C<sub>4:0</sub> + C<sub>6:0</sub> + C<sub>8:0</sub>) (Woo *et al.*, 1984; Arbige *et al.*, 1986; Lin and Jeon,

1987; McNeill and Connolly, 1989). For some non-Cheddar varieties, Kuzdzal-Savoie (1975) concluded that the concentration of caproic acid ( $C_{6:0}$ ) is the best guide to the lipolysis status of a cheese. She determined the following caproic acid levels (in mg/100 g dry matter) beyond which organoleptic defects was evident: Emmental, 8; Gruyère, 14; and Camembert, 20–25. For good quality Cheddar cheese, Urbach (1993) concluded that less than 0.52% of the fat is lipolysed at manufacture but up to 1.6% after ripening for 20 months. Cheeses that lay above this concentration had off-flavors; many of them were soapy, but oniony and metallic/vomit flavors also occurred. The percentage lipolysis was calculated from the amount of free  $C_{16:0}$  that had been released.

#### 15.4.2. Technological Consequences

While flavor defects are the most likely result of lipolysis in dairy products, several other practical problems may arise from an elevated level of FFAs. The most common of these is lack of foaming of pasteurised milk for cappuccino-style coffee (IDF, 1987). Reduced efficiency of skimming of raw milk and reduced churning efficiency in cream may be associated with lipolysis, especially where excessive agitation or pumping causes damage to the milk fat globule membrane.

Lipolysis in milk reduces its surface tension (Tarassuk and Smith, 1940) and, as a consequence, lipolysed milk has a low foaming capacity (Brunner, 1950; Dunkley, 1951). This is particularly noticeable during the steam-frothing of milk used for making cappuccino coffee, where stable foam formation is essential (Buchanan, 1965). Milk with an FFA level greater than 1.5 meq/l usually has poor foaming properties, while that with a level greater than 2 meq/l exhibits negligible steam-frothing. Commercial milk-processing operations, pasteurization and homogenization, markedly enhance the steam-frothing capacity of milk (Deeth and Smith, 1983). The reduction in surface tension and the consequent detrimental effect on foaming caused by lipolysis is believed to be due to the partial glycerides, particularly the monoglycerides, formed during lipolysis (Brunner, 1950; Buchanan, 1965).

Difficulties have been experienced in churning cream from spontaneously-rancid milk (Krukovsky and Sharp, 1936). The cream foams excessively and may take several times longer than normal cream to churn. Fat lost in the buttermilk is similar to that for normal milk (Fouts and Weaver, 1936). However, milk in which lipolysis is induced by agitation at a warm temperature gives higher fat losses on separation (Aule and Worstorff, 1975) due to the partial homogenization of the fat. Similarly, excessive pumping of milk has been found to reduce the fat content of cheese due to the loss of fat

in the whey (McDonald *et al.*, 1986). However, Hicks *et al.* (1990) attributed the reduced yield of cheese made from excessively pumped milk to loss in the whey of soluble FFAs produced by lipolysis rather than to the loss of fat. Such a situation is unlikely to arise from normal pumping in factories.

A high level of FFAs may cause minor errors in determination of the fat content of milk (e.g., a decrease of 0.01–0.03% fat per 1 meq/100 g increase in FFA), whether by older methods such as Röse-Gottlieb or turbidimetric or infrared methods (van Reusel, 1975; van de Voort *et al.*, 1987). Lipolysis may also lead to false positives in testing for antibiotic residues in milk (Carlsson and Björck, 1992).

## 15.5. Beneficial Effects of Lipolysis in Milk and Milk Products

### 15.5.1. Production of Desirable Flavor

Lipolysis plays an important role in providing the characteristic flavor of many milk products. In particular, the ripening of most cheese varieties is accompanied by lipolysis due to microorganisms or to added enzyme preparations, and, in raw milk cheese, to the milk LPL. Lipolysis is not extensive, but is more pronounced in some cheeses (e.g., blue-veined and hard Italian varieties), than in others. Excessive lipolysis renders the cheese unacceptable (Fox and Law, 1991; Gripon *et al.*, 1991).

In some mold-ripened cheeses, a very high FFA content (up to 25% of total fatty acids; Gripon, 1987) is acceptable [e.g., >66 000 mg/kg for Blue cheese (Horwood *et al.*, 1981) compared to <4000 mg/kg for good quality Cheddar; (Bills and Day, 1964)]. High levels of butyric acid characterize Italian hard cheeses and certain pickled cheeses (Fox and Guinee, 1987), [e.g., up to 520 mg/kg for Greek Feta (Horwood *et al.*, 1981) and >3000 mg/kg for Romano (Woo and Lindsay, 1984)]. An imbalance in flavor constituents can, nevertheless, lead to undesirably rancid or goaty (C<sub>4:0</sub>–C<sub>8:0</sub>) or soapy (C<sub>10:0</sub>–C<sub>12:0</sub>) flavors in these cheeses (Woo and Lindsay, 1984).

In ripening Cheddar cheese, some of the butyric and most of the higher free fatty acids are formed by lipolysis of the milk fat, mainly by lipases of lactic acid bacteria (Chapman and Sharpe, 1981; Aston and Dulley, 1982). In Swiss varieties, acids can arise through the action of lipases of propionibacteria (Oterholm *et al.*, 1970) or through fermentation (Kleinhenz and Harper, 1997). Italian varieties, such as Provolone and Pecorino cheeses acquire their characteristic flavor from the action of pregastric esterases, traditionally from rennet paste used to coagulate the milk but now in the form of commercially prepared oral gland extracts (Fox and Guinee, 1987; Birschbach, 1992). In Blue cheeses such as Gorgonzola, Roquefort, and Stilton, the FFAs produced by *Penicillium roqueforti* lipase (and milk lipase

when raw milk is used) are important both as flavor agents *per se* and as precursors for the methyl ketones, which provide the peppery taste of such cheeses (Kinsella and Hwang, 1976). A similar function is served by *P. camemberti* lipase in surface-mold ripened cheeses such as Brie and Camembert. Lipase preparations (microbial and pregastric) have been used to enhance the flavor (Arnold *et al.*, 1975; Huang and Dooley, 1976) and accelerate the ripening of cheese, for example Cheddar (Arbige *et al.*, 1986), buffalo milk Gouda (Rajesh and Kanawjia, 1990) and Blue cheese (Jolly and Kosikowski 1975a) (reviewed by Fox, 1988).

Flavor preparations typical of particular varieties of cheese can be produced with the aid of lipases of appropriate specificities (Kilara, 1985). Such flavors are used in processed cheeses, dips and spreads (Jolly and Kosikowski, 1975b). Controlled lipolysis of milk fat is also used to produce creamy and buttery flavors for bakery and cereal products, confectionery (milk chocolate, fudge), coffee whiteners, and other imitation dairy products (Arnold *et al.*, 1975; Fox, 1980; Kilara, 1985).

### 15.5.2. Digestion of Milk Fat

Absorption of fat in the newborn, and particularly in premature infants, is much less efficient than in adults due to the relatively low output of lipase and bile salts from the pancreas of the infant. Intragastric lipolysis by milk BSSL and lingual or salivary lipases, the secretion of which is stimulated by suckling, appears to augment the pancreatic lipase system in the newborn (Hamosh, 1979; Hernell and Blackberg, 1994).

Although LPL activity is commonly present in the milk of mammalian species, its role in the digestion of milk fat by the young animal has not been demonstrated (Olivecrona *et al.*, 2003). Olivecrona *et al.* (2003) speculated that LPL may serve an auxiliary role by binding milk fat globules to the mucosa, binding to intestinal cell surfaces and transferring lipids into the cells; these roles may not be dependent on the hydrolytic activity of the enzyme.

The bile salt-stimulated lipase in human milk acts as a supplementary milk fat digestive enzyme. Its characteristics suit it for such a role (O'Connor and Cleverly, 1989). It is both stable and fully active under the conditions pertaining in the gastrointestinal tract of the newborn and its lack of specificity enables it to hydrolyze a range of acylglycerols and other esters of nutritional significance, such as retinyl and cholesteryl esters (Hernell, 1975, 1985). BSSL has been detected in the gastric and intestinal contents of infants following the ingestion of fresh human milk, and average fat absorption has been found to be higher than after feeding cows' milk formulae or heated human milk (Fredrikzon *et al.*, 1978; Williamson *et al.*, 1978). This

increase is particularly significant in pre-term infants, or those suffering from pancreatic insufficiency (Williamson *et al.*, 1978; Hamosh *et al.*, 1981).

Bernback *et al.* (1990) found that complete digestion of human milk triglycerides *in vitro* requires the concerted action of gastric lipase, pancreatic co-lipase-dependent lipase and BSSL. While BSSL contributes to the hydrolysis of triglycerides and diglycerides, its unique function is to hydrolyze monoglycerides to FFA and glycerol, which may be absorbed more readily (Hernell, 1975). However, this does not apply to palmitic acid (C<sub>16:0</sub>) which is poorly absorbed as the free acid and is absorbed as the 2-monoacylglycerol; about 70% of this acid is esterified at the *sn*-2 position of human milk triacylglycerols (Innis *et al.*, 1994). *In vivo* studies in the suckling dog and mouse suggest, however, a greater role for BSSL in intragastric lipolysis than predicted by *in vitro* experiments (Iverson *et al.*, 1991; Howles *et al.*, 1999). BSSL also has ceramidase activity, which breaks down the ceramide produced from sphingomyelin after its hydrolysis by an indigenous acid sphingomyelinase C (Nyberg *et al.*, 1998).

Kittens fed formula supplemented with purified human BSSL grew twice as fast as those on formula alone (Wang *et al.*, 1989) and the production and use of BSSL as a dietary supplement has been proposed (Tang and Wang, 1989).

Investigations on the protective effect of breast-feeding against giardiasis in infants have led to the discovery that raw human, gorilla, and grey-cheeked mangabey milk can destroy *Giardia lamblia in vitro* (Gillin *et al.*, 1991). The toxic factor has been identified as FFAs produced by BSSL (Reiner *et al.*, 1986). Hamosh (1998) suggested that the third lipase in human milk, platelet-activating factor acetylhydrolase, might play a role in preventing inflammatory reactions.

## 15.6. Analytical Methods

### 15.6.1. Free Fatty Acids

The quantification of all FFAs in milk or milk products presents a difficult analytical problem and has been the subject of a large number of research publications. Reviews of the methods have been published by Kuzdzal-Savoie (1980), an IDF/ISO/AOAC Group (Anderson *et al.*, 1991), de la Fuente and Juarez (1993), Joshi and Thakar (1994) and Collomb and Spahni (1995).

The FFAs range from the water-soluble, short-chain acids such as butyric (C<sub>4:0</sub>) and caproic (C<sub>6:0</sub>) to the water-insoluble, long-chain acids such as palmitic (C<sub>16:0</sub>) and stearic (C<sub>18:0</sub>). In milk and milk products they are accompanied by relatively large amounts of fat, largely triglycerides,

water-soluble acids such as lactic acid, and phospholipids, some of which are acidic.

The methods usually involve an initial step to isolate the fatty acids, with or without the milk fat, followed by a quantification step, with or without derivatisation of the acids. Direct methods using infrared (Buer-meyer *et al.*, 2001; Pedersen, 2003) or biosensor technology (Ukeda *et al.*, 1992) that do not require a pretreatment step offer some obvious advantages and may be suitable for in-line or at-line FFA monitoring. A summary of the methods used is given in Table 15.4.

The methods involving fat separation, of which the Bureau of Dairy Industries (BDI) methods are most common, measure only fat-soluble FFAs and hence underestimate the total FFA level (Deeth *et al.*, 1975; Van Crombrugge *et al.*, 1982). For milk, this is of little consequence as the level of the long-chain acids correlates highly with that of the short-chain acids responsible for the flavor. In products such as butter, measurement of fat-soluble FFAs only will not detect small increases in FFA postmanufacture which can have a detrimental effect on flavor due to the short-chain FFAs present (Deeth *et al.*, 1979).

Solvent extraction methods estimate a high proportion of FFAs. However, when the acids are quantified by titration, some of these methods can overestimate the FFA level because of the inclusion of lactic acid, acidic phospholipids and other acidic interfering compounds in the extract (Chilliard *et al.*, 1983; Selselet-Attou *et al.*, 1984). Solvent extraction methods based on mixtures of isopropanol, heptane and sulphuric acid (Dole and Meinertz, 1960; Landaas and Solberg, 1974; Deeth *et al.*, 1975; Mouillet *et al.*, 1981; Mahieu, 1983) have found widespread use in several countries and are used in routine analysis of large numbers of samples in automated versions (Lindqvist *et al.*, 1975; Suhren *et al.*, 1977; Bråthen, 1984; Anderson *et al.*, 1991).

Adsorption of FFAs on various solid supports, usually after solvent extraction, has led to the development of the best methods for quantifying all the FFAs present in a product. Some of these in which the FFAs are quantified by GC are now regarded as reference methods (Anderson *et al.*, 1991). In some earlier solid adsorption methods, hydrolysis of the fat occurred on the support and overestimated the true FFA level (Stark *et al.*, 1976). However, methods have been developed using deactivated alumina (Deeth *et al.*, 1983) or ion exchange resins (Needs *et al.*, 1983; Spangelo *et al.*, 1986; de Jong and Badings, 1990) in which fat hydrolysis does not occur.

Capillary gas chromatography of FFAs as either free acids (de Jong and Badings, 1990) or as methyl esters (Chilliard *et al.*, 1983; Juarez *et al.*, 1992) provides excellent resolution of the component acids. Similarly, well-resolved chromatograms have been obtained in HPLC analyses of milk FFAs as

**Table 15.4.** Summary of Methods Used to Determine Free Fatty Acids (FFAs)

Type of isolation	Isolation reagent/method	Method of quantification	Product	References	
Fat separation	Churning	Titration	Milk, cream	Herrington and Krukovsky (1939)	
	Melting	Titration	Butter, milk fat	Deeth and Fitz-Gerald (1976), IDF (1989)	
	Detergent demulsification (BDI)	Titration—manual	Milk, cream	Thomas <i>et al.</i> (1955)	
		Titration—semiautomatic	Milk	Driessen <i>et al.</i> (1977)	
Solvent extraction	Isopropanol–heptane–H <sub>2</sub> SO <sub>4</sub> (Dole)	Titration—photometric	Cream, whole milk powder	Evers <i>et al.</i> (2000)	
		Titration—manual colorimetric	Milk, cream	Deeth <i>et al.</i> (1975), Mouillet <i>et al.</i> (1981)	
	Isopropanol–heptane–H <sub>2</sub> SO <sub>4</sub> –isobutanol (Lipo-R)	Titration—automatic colorimetric	Milk	Suhren <i>et al.</i> (1977), Cartier <i>et al.</i> (1984)	
		Titration—automatic potentiometric	Milk	Bosset <i>et al.</i> (1990)	
	Ether–heptane–ethanol–H <sub>2</sub> SO <sub>4</sub> + weak anion exchanger	Titration—manual	Milk	Mahieu (1983)	
		Capillary GC of FFAs	Milk, cheese	de Jong and Badings (1990)	
	Ether–hexane–HCl	Titration	Milk	Salih <i>et al.</i> (1977)	
		Chloroform–heptane–methanol	Colorimetry of copper soaps	Milk, cheese	Koops and Klomp (1977), Shipe <i>et al.</i> (1980), Bynum <i>et al.</i> (1984)
	Acetonitrile–H <sub>2</sub> SO <sub>4</sub> + CH <sub>3</sub> I–strong anion exchanger	Diethyl ether and acid + tetramethylammonium hydroxide	GC of methyl esters	Milk	Spangelo <i>et al.</i> (1986)
			GC of methyl esters after pyrolysis of the tetramethylammonium soaps	Cheese	Juarez <i>et al.</i> (1992), Chavarrri (1997)
Benzene–K oxalate–H <sub>3</sub> PO <sub>4</sub>		Colorimetry using Rhodamine 6G	Milk	Nakai <i>et al.</i> (1970)	

(Continued)

Table 15.4. (Continued)

Type of isolation	Isolation reagent/method	Method of quantification	Product	References
	Alcohol-ether-petroleum ether	Titration	Milk	Frankel and Tarassuk (1955), Pillay <i>et al.</i> (1980)
Solid phase adsorption	Silicic acid-H <sub>2</sub> SO <sub>4</sub> -5% butanol in chloroform Amberlyst 26	Titration—manual GC of methyl esters	Milk, cream Milk, cream, butter	Harper <i>et al.</i> (1956) Needs <i>et al.</i> (1983), McNeill <i>et al.</i> (1986)
	Dowex 1 × 2 Aminopropyl	GC of methyl esters Capillary GLC of FFAs	Milk Milk, cheese, yogurt	Spangelo <i>et al.</i> (1986) de Jong and Badings (1990), de Jong <i>et al.</i> (1994), Chavarri (1997)
	Amberlite IRA-400 Silicic acid-KOH Alumina-deactivated	GC of methyl esters GC of FFAs GC of FFAs	Milk, cream, butter Milk Milk, milk fat, Butter, cheese, milk powder	Kintner and Day (1965) Woo and Lindsay (1980) Deeth <i>et al.</i> (1983)
Nil	Enzymatic Derivatization Derivatization	Colorimetry—manual, automatic Fluorimetry HPLC of 2-nitrophenylhydrazides HPLC of <i>p</i> -bromophenacyl esters	Milk Human milk Milk, butter, cheese Butter, milk fat	Koops <i>et al.</i> (1990) Christmass <i>et al.</i> (1998) Miwa and Yamamoto (1990) Reed <i>et al.</i> (1984), Garcia <i>et al.</i> (1990)
	Methyl urea + BF <sub>3</sub> -methanol	GC of methyl esters Infra Red Flow injection analysis with microbial electrode	Ghee Milk Milk	Sharma and Bindal (1987) Buermeier <i>et al.</i> (2001), Pedersen (2003) Ukeda <i>et al.</i> (1992)

GC = Gas chromatography

HPLC = High-performance liquid chromatography

*p*-bromophenacyl esters (Garcia *et al.*, 1990) or 2-nitrophenylhydrazides (Miwa and Yamamoto, 1990). Thus, it is now possible to obtain accurate data for the individual FFAs in milk and milk products.

Some methods which do not involve separation of the FFAs from the milk fat or the whole product have considerable appeal because of their simplicity. Sharma and Bindal (1987) exploited the property of methyl urea to complex triglycerides in producing methyl esters with BF<sub>3</sub>-methanol without first separating the FFAs from the fat, while Spangelo *et al.* (1986) were able to methylate FFAs in an acetonitrile extract of milk with methyl iodide in the presence of an anion exchange resin as catalyst. Miwa and Yamamoto (1990) derivatised the FFAs in milk and milk products for HPLC analysis by direct reaction with 2-nitrophenylhydrazine hydrochloride.

The rapid enzymatic method of Koops *et al.* (1990) involving acyl-CoA synthetase and acyl-CoA oxidase and the colorimetric measurement of the resulting hydrogen peroxide have the potential to become routine procedures. They have been automated and showed good agreement with the BDI method on farm milk samples. In another enzymatic method, Christmass *et al.* (1998) linked acyl-CoA synthetase, UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate-1-dehydrogenase, and the NADH-luciferase to determine FFAs in human milk. The fluorimetric measurement of the resultant NADH overcame the problem of cloudiness due to the added milk, which affects colorimetric measurements.

Several publications have compared various methods for determining FFAs in milk and dairy products (Van Crombrugge *et al.*, 1982; Chilliard *et al.*, 1983; Suhren, 1983; Bråthen, 1984; Cartier *et al.*, 1984; Selselet-Attou *et al.*, 1984; Ikins *et al.*, 1988; Anderson *et al.*, 1991; Joshi and Thakar, 1994). In general, high correlations were found between the various methods although different methods suffered from different limitations such as overestimation due to the inclusion of lactic acid or underestimation of short-chain acids.

Thus, the choice of method depends on the application. For routine analyses of FFAs, the BDI method or modifications of it, and methods based on the Dole extraction procedure appear to be most popular (IDF, 1983), while for accurate determination of all FFAs in a product, the capillary GC (Chilliard *et al.*, 1983; de Jong and Badings, 1990; Anderson *et al.*, 1991) or the HPLC methods (Garcia *et al.*, 1990) are the methods of choice. Solvent extraction using acidified ether (Salih *et al.*, 1977) followed by separation of the free acids from fat on an anion exchange resin, [e.g., Amberlyst 26 (Needs *et al.*, 1983)], and capillary gas chromatography of the acids or their methyl esters has been suggested by the IDF as a reference method (Anderson *et al.*, 1991).

Most of the methods discussed above have been used with milk but the majority can be applied to other products. Some minor variations in extraction procedures, such as the inclusion of anhydrous sodium sulphate to remove water (Deeth *et al.*, 1983) or inclusion of an aqueous acid wash step to remove lactic acid (Chilliard *et al.*, 1983) may be necessary. Collomb and Spahni (1995) suggested that the above International Dairy Federation reference method could be a universal method if the adaptations made by McNeill *et al.* (1986) for butter and McNeill and Connolly (1989) for cheese were incorporated.

### 15.6.2. Lipase Activity

Numerous methods have been used to measure the activity of lipases from various sources (Jensen 1983; Thomson *et al.*, 1999; Deeth and Touch, 2000; Chen *et al.*, 2003). They vary considerably in the substrate used, the form of the substrate, additives to the assay mix and the method for determining the extent of hydrolysis.

The natural substrates for lipases are triglycerides but, because of the complexity of these and the fact that they seldom contain a chromophore or other label to enable ready detection of the products, several synthetic substrates have been developed. These enable different detection techniques such as spectrophotometry, fluorimetry, chromatography, or radiometry to be used. It is important to note that, by definition, true lipases are active only on water-insoluble esters while esterases cleave only water-soluble esters (Jaeger *et al.*, 1994). Thus, it is important that methods used for milk and milk products use substrates, which detect true lipase but not esterases as lipases play a major role in the hydrolysis of milk fat, while the role of esterases is considered insignificant (McKay *et al.*, 1995).

Since lipases act on lipids at lipid–water interfaces, preparation of substrates in a suitable physical form for maximal lipase activity is very important. Preparation methods include: emulsification with an emulsifying agent; incorporation into a gel; dissolution in a water-soluble organic solvent, such as 2-methoxyethanol or tetrahydrofuran, followed by addition to an aqueous reaction mixture; sonication, with or without emulsifier; and formation of a thin film or monolayer.

The most common form of a lipid substrate is as an emulsion, stabilized by a surface-active agent, including bile salts, and gums. Regardless of the method used, the formation of a good emulsion is essential, since the rate of the lipase reaction is dependent on the surface area of the substrate at the substrate–water interface. This can be achieved by vigorous shaking, blending, sonication, or homogenization of the substrate in the aqueous medium. However, although an apparently fine emulsion can be produced by these

methods, the available lipid surface area will differ among methods due to differences in the physico-chemical properties of the substrate system. Accordingly, comparison of lipase activity data from reports using different analytical methods is difficult.

The emulsified water-insoluble substrate is usually incubated with a buffered aqueous enzyme preparation. Lipase activity can be determined by continuous measurement of the reaction products or by incubating for a set time and determining the total amount of product formed, or substrate used. One unit (U) of lipase activity is usually defined as the amount of enzyme required to liberate a certain quantity (e.g., 1  $\mu\text{mol}$ ) of product in a certain time (e.g., 1 min) under given conditions.

A current challenge in dairy chemistry is the measurement of low levels of bacterial lipases, which can cause defects in milk and milk products, particularly during prolonged storage (IDF, 1983; Stead, 1989; Deeth and Touch, 2000). To this end, many of the sensitive methods described above are applicable to detecting small amounts of FFAs released during incubation of the enzyme source with triglyceride substrates. In order to maximise the sensitivity of assays for bacterial enzymes, long incubations at temperatures at which the indigenous LPL is not active ( $\geq 45^\circ\text{C}$ ) can be used (Mortensen and Jansen, 1982).

In addition, colorimetric assays using  $\beta$ -naphthyl caprylate (McKellar and Cholette, 1986; Versaw *et al.*, 1989) or *p*-nitrophenyl esters (Blake *et al.*, 1996; Bendicho *et al.*, 2001) as substrates, as well as fluorimetric assays using 4-methylumbelliferyl esters (Stead, 1983; 1984) have been proposed as sensitive methods for detecting bacterial enzymes in milk or milk products. However, problems such as non-enzymic hydrolysis (McKellar, 1986), interference by milk fat and milk proteins (Stead, 1983), and poor correlations of activities measured with those on milk fat (Fitz-Gerald and Deeth, 1983) have limited their use in predicting the lipolytic stability of milk products during storage. Assays based on umbelliferyl esters (de Laborde de Monpezat *et al.*, 1990) have been shown to have advantages over those using 4-methylumbelliferyl esters and may find application in milk and milk products.

Some progress has been made towards developing a sensitive ELISA method for bacterial lipases (Stepaniak *et al.*, 1987b). However, this technique detects the enzyme protein rather than its activity and hence may be of little practical value to the dairy industry. Further developments can be expected in this field.

The more common methods used for milk and bacterial lipases are summarized in Table 15.5.

Deeth and Touch (2000) evaluated the suitability of various methods for dairy applications. They considered these in two categories: screening

**Table 15.5.** Methods for Determining Lipase Activity in Milk and Milk Products (after Deeth and Touch, 2000)

Method	Substrate	Assay components/ Conditions	Measurement	Applications	References
1. Titrimetric					
(a) pH-stat					
	Tributyrin, olive oil	NaCl	Continuous potentiometric titration	Milk lipase, bacterial lipase	Parry <i>et al.</i> (1966), Egelrud and Olivecrona (1973), Andersson <i>et al.</i> (1979), Makhzoum <i>et al.</i> (1996)
	Tributyrin; Triolein Olive oil/triolein	NaCl Sonication before and during assay	- -	Bacterial lipase Bacterial lipase	Tomasini <i>et al.</i> (1993) Linfield <i>et al.</i> (1985), Yang <i>et al.</i> (1994)
	Tributyrin	Na taurocholate	-	BSSL	Jubelin and Boyer (1972)
(b) Solvent-extraction titration methods					
	Milk fat	Blood serum, heparin, CaCl <sub>2</sub>	Extraction with isopropanol–heptane–H <sub>2</sub> SO <sub>4</sub> , titration to visual endpoint	Milk lipase	Deeth and Fitz-Gerald (1977)
	Milk fat		Extraction with isopropanol–heptane–H <sub>2</sub> SO <sub>4</sub> , titration to visual endpoint	Bacterial lipase	Fitz-Gerald and Deeth (1983)
	Tributyrin	NaCl	Extraction with ether-petroleum ether, titration to visual endpoint	Milk lipase Bacterial lipase	Castberg <i>et al.</i> (1975) Fitz-Gerald and Deeth (1983)
2. Colorimetric					
(a) $\beta$ -Naphthol methods					
	$\beta$ -Naphthyl caprylate	Thiomersal, bile salts, Ca	Extraction of $\beta$ -naphthol-Fast Blue complex with ethyl acetate, spectrophotometry at 540 nm	Bacterial lipase	McKellar and Chollette (1986)

(Continued)

**Table 15.5.** (Continued)

Method	Substrate	Assay components/ Conditions	Measurement	Applications	References
	$\beta$ -Naphthyl caprylate	Thiomersal, bile salts, Ca	Production of $\beta$ -naphthol-Fast Blue complex, clarification with ethanol-ethyl acetate, spectrophotometry at 540 nm	Bacterial lipase	Versaw et al. (1989)
(b) p-Nitrophenol methods					
	<i>p</i> -Nitrophenyl caprylate	Sodium deoxycholate	Acetone used to clarify the solution of substrate,	Bacterial lipase	Owusu et al. (1992), Makhzoum et al. (1996)
	<i>p</i> -Nitrophenyl caprylate		spectrophotometry at 410 nm	Bacterial lipase	Blake et al. (1996), Bendicho et al. (2001)
	<i>p</i> -Nitrophenyl acetate	Na taurocholate, acetonitrile	Reflectance colorimetry (b* color); spectrophotometry at 418 nm	BSSL	Swan et al. (1992)
	<i>p</i> -Nitrophenyl butyrate	Acetonitrile	Spectrophotometry at 418 nm	Bacterial lipase	Humbert et al. (1997)
(c) Indigo methods					
	Indoxyl acetate and caprylate		Addition of Clarifying Agent <sup>®</sup> , spectrophotometry at 420 nm	Bacterial lipase	Slack (1987), Allen (1994), Munnich and Haasmann (1999), Brand et al. (2000)
3. Fluorimetric					
(a) 4-Methyl umbelliferone methods					
	4-Methyl-umbelliferyl esters (in 2-methoxy ethanol)	Bile salt, cetrinide	Colorimetry of indigo formed	Bacterial lipase	Stead (1983, 1984), Fitz-Gerald and Deeth (1983), Vercet et al. (1997)
			Fluorimetry at 450 nm (emission), 360 nm (excitation)		

(Continued)

Table 15.5. (Continued)

Method	Substrate	Assay components/ Conditions	Measurement	Applications	References
(b) Umbelliferone methods					
	Umbelliferyl myristate		Fluorimetry at 470 nm (emission), 360 nm (excitation)	Bacterial lipase	de Laborde de Monpezat <i>et al.</i> (1990)
(c) Substituted triacylglycerol methods					
	1,2-DPPBA <sup>b</sup>	Na taurocholate	Fluorimetry at 340 (excitation), 396 nm (emission)	Bacterial lipase	Celestino <i>et al.</i> (1996)
4. Chromatographic					
	Triacylglycerols (esp. triolein)		Chloroform-methanol extraction, HPLC with detection at 208 nm; GC of derivatized or free fatty acids.	Bacterial lipase	Veeraragavan (1990), Choi and Jeon (1993)
5. Radiometric					
	Radiolabelled triacylglycerols		Free fatty acid isolation by solvent partitioning or chromatography, scintillation counting of labelled fatty acids	All lipases	Clegg (1980)
	<sup>3</sup> H-triolein-gum acacia	Na taurocholate, serum albumin, NaCl	Extraction with CHCl <sub>3</sub> -methanol-heptane, scintillation counting of <sup>3</sup> H-oleic acid	BSSL	Hernell and Olivecrona (1974b)
	Triacylglycerols (e.g., tributyrin)	In agar or as liquid emulsion	Measure size of clear zone or decrease in turbidity (decrease in absorbance at e.g., 450 nm)	All lipases	Lawrence <i>et al.</i> (1967), Smeltzer <i>et al.</i> (1992), Choi <i>et al.</i> (1994)

<sup>a</sup> BSSL = bile-salt-stimulated lipase.<sup>b</sup> 1,2-DPPBA = 1(3)-pyrenyl/butanoyl-2,3(1,2)-dipalmitoyl-*sn*-glycerol.

tests and confirmatory tests. The former can accommodate large numbers of samples in a relatively short time but may result in some false positives or false negatives while the latter, which involve the use of natural triglyceride substrates, often milk fat, are generally more time-consuming. The most favored methods for each of these categories are as follows:

1. Screening tests:

- Fluorimetric and colorimetric assays using non-triacylglycerol substrates,
- Titrimetric assays using tributyrin,
- Agar diffusion assays using tributyrin, triolein or milk fat.

2. Confirmatory tests:

- Chromatographic assays based on triolein or milk fat as substrate,
- Titrimetric assays based on triolein or milk fat as substrate.

## 15.7. Prevention of Hydrolytic Rancidity

Sufficient knowledge exists to enable dairy industry personnel to take precautions to ensure a low incidence of hydrolytic rancidity and associated problems in milk and milk products. Recommendations for prevention have been published (Deeth and Fitz-Gerald, 1976; IDF, 1991; O'Brien *et al.*, 1996). A summary of these is given below.

1. For problems associated with milk lipase:

- Avoid large numbers of cows in late lactation, especially under poor feed conditions;
- Provide a constant balanced diet for the cows;
- Design, install and maintain milking equipment correctly;
- Avoid excessive air intake at teat cups;
- Minimize centrifugal pumping, especially of warm milk and with air incorporation;
- Avoid agitation of raw milk, particularly using air;
- Never mix raw and homogenized milk.

2. For problems associated with microbial lipases:

- Do not store milk, either raw or pasteurised, for a long period;
- Do not store milk or milk products at an inappropriately high temperature;
- Ensure that all milk handling and processing equipment is properly cleaned and sanitized.

Hygiene on the farm and in the factory is of paramount importance in controlling microbial growth and minimising lipolysis problems. Inadequately cleaned equipment can be a major source of lipolytic psychrotrophic contaminants (Drew and Manners, 1985; Stead, 1987).

It has been suggested that some form of heat treatment, either thermization (Humbert *et al.*, 1985; Matselis and Roussis, 1998) or HTST pasteurization (Mogensen and Jansen, 1986), of milk on arrival at the factory should be performed to minimise the incidence or reduce the severity of lipolysis problems. While such treatments have been shown to be effective, they increase the cost of processing and result in double heat treatment of milk, which is not permitted in some countries. Carbonation of raw milk (with 30 mM CO<sub>2</sub>) has also been reported to reduce the growth of lipolytic psychrotrophs and also to reduce the level of FFAs in cheese made from the carbonated milk (McCarney *et al.*, 1994).

Once lipolysis has occurred there is little that can be done to reduce its effects on quality, although, in laboratory trials, Nakai (1983) successfully removed FFAs from rancid milk by adsorbing them on activated charcoal and Takacs *et al.* (1989) by the use of a C18 column. However, such nonspecific adsorption methods are unlikely to find widespread use as other milk components such as proteins and vitamins are also removed.

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# Lipid Oxidation

T.P. O'Connor and N.M. O'Brien

## 16.1. Introduction

Lipid oxidation is one of the most basic chemical reactions that occur in food, generally resulting in deterioration in sensory and nutritional quality. Many reviews of the chemistry of lipid oxidation have been published (Labuza, 1971; Frankel, 1980, 1982, 1985, 1988, 1991, 1998; Larel, 1980; Schaich, 1980; Richardson and Korycka-Dahl, 1983; Porter, 1986; Chan, 1987; Grosch, 1987; Gardner, 1989; Kochhar, 1996; Min and Lee, 1996; St. Angelo, 1996; Belitz and Grosch, 1999; Kolakowska, 2003). Lipid oxidation is essentially a free-radical chain reaction involving initiation, propagation and termination stages. Unsaturated fatty acids are oxidized to form odorless, tasteless hydroperoxides. These are unstable and degrade to yield flavorful carbonyls and other compounds. Frankel (1998) has reviewed recent advances in the understanding of the chemistry of autoxidation. Inhibiting the progress of lipid oxidation in foods, including milk and milk products, is a key factor in maintaining quality and extending shelf-life.

Milk is a complex biological system containing many factors, which may act as antioxidants and/or pro-oxidants. The relative amounts of these factors in milk are influenced by parameters such as the breed, health, nutritional status, and stage of lactation of the cow. Subsequent processing and storage of milk may also exert a profound influence on the progress of lipid oxidation. The objective of this chapter is to review the many factors, both indigenous and exogenous, that influence lipid oxidation in milk, the flavor consequences, and the measurement of lipid oxidation in milk fat.

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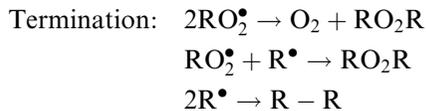
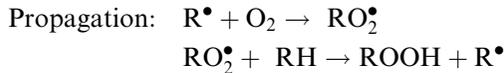
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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

## 16.2. Mechanism of Lipid Autoxidation

The hydroperoxide theory of the oxidation of unsaturated lipids is universally accepted. The fundamental principles were elucidated by the work of Farmer *et al.* (1942), Bolland and Gee (1946) and Bateman *et al.* (1953). The initial step in the autoxidation of unsaturated fatty acids is the formation of free radicals. The formation of the initial free radical to start the oxidation process may be due to factors such as irradiation, metal complexes, enzymes or active oxygen species. In the case of monounsaturated and nonconjugated polyunsaturated fatty acids in milk lipids, the reaction is usually initiated by removal of a hydrogen from the methylene group adjacent to the double bond. The resulting free radical reacts with ground-state molecular oxygen to form a peroxide free radical. This, in turn, reacts with another unsaturated molecule to continue the chain reaction and generate a hydroperoxide. A sequence involving initiation, propagation and termination reactions has been proposed to explain the autoxidation of lipids:



In general, free radical chain reactions proceed with a very low overall activation energy (Waters, 1971). However, in foods, such as butter, the rate of oxidation may be as much a feature of their microscopic structure which affects diffusion of oxygen, as of their chemical composition.

Schaich (1980) proposed the following generalizations:

1. The rate of oxidation is directly proportional to the amount of peroxide produced and at low oxygen pressures, to the concentration of oxygen.
2. As the concentration of oxygen increases, its influence on oxidation rate decreases. At atmospheric pressure, the rate of oxidation is independent of oxygen concentration.
3. In the early stages of oxidation, the concentration of  $\text{RO}_2\text{H}$  may be very low. Initiating events other than the homolytic decomposition of hydroperoxides are critical at this early stage. Once decomposition of  $\text{RO}_2\text{H}$  has occurred, the rate of hydrogen abstraction from

unsaturated lipids by the resultant alkoxy radicals ( $\text{RO}^\bullet$ ) is in the order of  $10^4$ – $10^6$  times faster than by peroxy radicals ( $\text{RO}_2^\bullet$ ) generated in the propagation phase. Thus, the production of peroxides mediates the rate of lipid oxidation, and the stability of peroxides, as affected by food constituents, is a key factor influencing the rate of development of oxidative rancidity.

One key role of pro-oxidant metals (e.g., copper, iron, haem proteins) in promoting oxidative rancidity is their capacity to catalyse the decomposition of pre-formed hydroperoxides to initiate new oxidation chains (Korycka- Dahl and Richardson, 1980). Hydroperoxides are relatively unstable and enter into numerous and complex breakdown and interaction mechanisms responsible for the production of myriad compounds of various molecular weights, flavor thresholds and biological significance (Nawar, 1985; Chan, 1987; Grosch, 1987). In addition to autoxidation, lipid oxidation can proceed along a photooxidation route or a lipoxygenase route. These differ from autoxidation at the initiation stage only.

Food lipids possess an inherent stability to oxidation, which is influenced by the presence of antioxidants and pro-oxidants. After a period of relative stability (induction period), lipid oxidation becomes autocatalytic and rancidity develops. Thus, the typical time-course of autoxidation, as measured by the concentration of hydroperoxides, consists of a lag phase (induction) followed by the rapid accumulation of hydroperoxides, which reaches a maximum and then decreases as hydroperoxide decomposition reactions become more important. The longer the induction period, the more stable the food to oxidation (Lundberg, 1962).

### 16.3. Oxidation Products and Off-Flavors

Milk is characterized as having a pleasing, slightly sweet taste with no unpleasant after-taste (Bassette *et al.*, 1986). However, its bland taste makes it susceptible to a variety of flavor defects. Autoxidation of unsaturated fatty acids gives rise to unstable hydroperoxides, which decompose to a wide range of carbonyl products, many of which can contribute to off-flavors in dairy products. The principal decomposition products of hydroperoxides are saturated and unsaturated aldehydes (Frankel *et al.*, 1961), with lesser amounts of unsaturated ketones (Stark and Forss, 1962), saturated and unsaturated hydrocarbons (Forss *et al.*, 1961), semialdehydes (Frankel *et al.*, 1961) and saturated and unsaturated alcohols (Hoffman, 1962; Stark and Forss, 1966).

In addition to those carbonyls that are theoretically possible from the degradation of the hydroperoxides of the principal unsaturated fatty acids in

milk, others have been isolated and identified. This suggests that further oxidation of unsaturated aldehydes initially formed, migration of double bonds or isomerization may occur during autoxidation (Weihrauch, 1988). Since milk fat contains many minor unsaturated fatty acids, very many carbonyl products may be produced during autoxidation. Hence, the overall flavor produced during the autoxidation of milk fat is a combination of many flavors imparted by individual carbonyls present at minute concentrations. Patton *et al.* (1959) demonstrated that 2,4-decadienal imparts an oily, deep-fried, off-flavor in aqueous solutions at concentrations less than 0.5 µg/kg.

It is difficult, however, to correlate specific off-flavors in dairy products with specific carbonyls or groups of carbonyls owing to: (1) the multitude of compounds produced; (2) difficulties arising in qualitative analyses of oxidized dairy products; (3) differences in the threshold value of individual compounds; (4) similarity of flavors imparted by individual compounds near their flavor thresholds; (5) a possible additive and/or antagonistic effect, with regard to both flavor and threshold values of mixtures of compounds; (6) the possible existence of a compound or groups of compounds not previously identified; and (7) the difficulties involved in adding pure compounds to dairy products as a means of evaluating their flavor characteristics (Weihrauch, 1988).

Despite the above difficulties, several specific chemicals have been associated with specific off-flavors in dairy products. Forss *et al.* (1955a,b) reported that *n*-hexanal, 2-octenal, 2-nonenal, 2,4-heptadienal and 2,4-nonadienal are the principal carbonyls contributing to the copper-induced "cardboard" off-flavor in milk. Hall and Lingnert (1986) associated this flavor defect with *n*-hexanal in spray-dried whole milk. 1-Octen-3-one has been associated with a "metallic" off-flavor in dairy products (Stark and Forss, 1962), the metallic off-flavor being reproduced by addition of 1-octen-3-one to milk or cream (Bassette *et al.*, 1986). 1-Octen-3-one has a threshold concentration of 1 µg/kg in butterfat (Shipe *et al.*, 1978).

"Creamy" flavors in butter have been associated with 4-*cis* heptenal produced for autoxidation of isolinoleic acid (Begeman and Koster, 1964). "Drier" flavor in foam spray-dried milk has been associated with 6-*trans*-nonenal, which has a flavor threshold in fresh milk of 0.07 µg/kg (Parks *et al.*, 1969). Bassette and Keeney (1960) implicated a homologous series of autoxidation-derived saturated aldehydes, together with products of Maillard browning, in "cereal-type" off-flavors in powdered skim milk. "Staleness" in dry whole milk may be associated with saturated and unsaturated aldehydes (Parks and Patton, 1961). 2,4-Decadienal has been reported to be the principal compound responsible for the off-flavor associated with spontaneously oxidized milk (Parks *et al.*, 1963). Oxidized flavors in sunlight-exposed milk are commonly related to C<sub>6</sub> to C<sub>11</sub> alk-2-enals

(Wishner and Keeney, 1963). “Fishy” flavor in milk fat is due to a mixture of 1-octen-3-one, the compound associated with metallic flavor, and an “oily” fraction containing *n*-heptanal, *n*-hexanal, 2-hexenal and heptan-2-one (Forss *et al.*, 1960a,b). Some 40 volatile compounds were identified in cold-stored cultured butter with a fishy off-flavor, including 4-*cis*-heptenal, 2-*trans*, 4-*cis*-decadienal, 2-*trans*, 6-*cis*-nonadienal, 2,2,7-decatrienal, 3-*trans*, 5-*cis*-octadien-2-one, 1-octene-3-one and 1-octen-3-ol. “Cucumber” flavor has been associated with 2,6- and 3,6-nonadienal and “mushroom” flavor with 1-octen-3-ol (Badings and Neeter, 1980).

Forss *et al.* (1960a,c) compared the qualitative and quantitative distribution of carbonyl compounds in dairy products with “fishy,” “tallowy” or “painty” off-flavor. Total content of volatile carbonyl compounds was approximately 10 times greater in the “tallowy” and 100 times greater in the “painty” butterfat than in “fishy” butterfat. “Tallowy” butterfat contained greater amounts of *n*-heptanal, *n*-octanal, *n*-nonanal, 2-heptanone 2-heptenal and 2-nonenal, while “painty” butterfat contained greater amounts of *n*-pentanal and C<sub>5</sub> to C<sub>10</sub> alk-2-enals.

Flavor threshold values for carbonyl compounds are influenced by factors such as the number of carbon atoms, degree and location of unsaturated double bonds, isomerism, additive and/or antagonistic effects of mixtures of carbonyls and the medium in which the carbonyls exist (Day *et al.*, 1963; Meijboom, 1964). Many carbonyls have up to 100 times greater flavor potency in an aqueous medium (e.g., liquid milk) than in a fat or oil (e.g., butteroil). Hence, off-flavor tends to be noticeable at lower concentrations of carbonyl compounds in liquid milk than in butter.

Oxygen and light (particularly ultraviolet wavelengths) exert a synergistic effect on fat degradation with resulting off-flavor and rancidity (IDF, 1982). The sensitivity of cream to light is influenced by heat treatment (e.g., off-flavor develops more rapidly in raw or pasteurized cream than in UHT cream). Free sulphhydryl groups, exposed upon denaturation of whey proteins in UHT cream, are thought to inhibit off-flavor development (Bull, 1992). Oxidative deterioration of UHT milk is enhanced by increased levels of dissolved oxygen and increased storage temperature (Jeon *et al.*, 1978; Adhikari and Singhal, 1992). The chemical composition of the volatile carbonyl compounds produced due to light-induced lipid oxidation has been reported to be different from that produced due to copper-induced oxidation (Jenq *et al.*, 1988).

### 16.3.1. Spontaneous Oxidation in Milk

Off-flavor due to spontaneous oxidation of milk fat is a troublesome issue because the process and its prevention are not well understood and it tends to occur in otherwise well-managed, high-yielding dairy herds

(Barrefors *et al.*, 1995; Granelli *et al.*, 1998). Dunkley and Franke (1967) classified milk into three categories based upon its susceptibility to oxidation:

1. Spontaneous milk is capable of developing oxidized flavor within 48 h of milking without the presence of contaminating iron or copper. Bruhn *et al.* (1976) reported that 12–20% of raw milk samples are in this category.
2. Susceptible milk does not oxidize spontaneously but does develop oxidized flavor following contamination with iron or copper. Use of noncorrodible dairy equipment has reduced the incidence of copper contamination.
3. Non-susceptible milk does not oxidize even in the presence of iron or copper.

Spontaneous oxidation of milk fat, which has been known for over 60 years (Corbett and Tracy, 1943), is influenced by heredity, stage of lactation and feeding practices (Shipe, 1964). Some cows consistently produce spontaneous milk, others occasionally, and others not at all (Parks *et al.*, 1963). Differences between milk from the different quarters of the same cow may occur.

Aurand and Woods (1959), Astrup (1963) and Aurand *et al.* (1967, 1977) proposed that spontaneous oxidation in milk is directly related to Xanthine oxidoreductase activity. This enzyme has iron, molybdenum and flavin cofactors and is a major component of the fat globule membrane. While some evidence suggests that Xanthine oxidoreductase is involved to an extent in the oxidation of milk lipids (Allen and Humphries, 1977; Hill, 1979; Allen and Wreiden, 1982b), others have reported no relationship between spontaneous oxidation and Xanthine oxidoreductase activity (Smith and Dunkley, 1960; Rajan *et al.*, 1962). King (1958) noted that spontaneous milks had a higher total copper concentration in the fat globule membrane than milks classified as susceptible or resistant. Smith and Dunkley (1960) hypothesized that endogenous copper in milk formed a complex with ascorbate, which was involved in spontaneous oxidation of milk lipids. Aurand *et al.* (1977) suggested that a combination of light, copper and Xanthine oxidoreductase generated singlet oxygen, which then initiated the oxidative process. Hill *et al.* (1977) proposed that oxidation is induced by copper in a pathway involving  $\bullet\text{OH}$  radicals, while Xanthine oxidoreductase in the fat globule membrane and lactoperoxidase in the milk serum were involved in a secondary pathway generating singlet oxygen with consequent oxidation of milk lipids. Hill (1979) suggested a coupled enzyme system in which lactoperoxidase catalyses lipid oxidation generating aldehyde substrates for Xanthine oxidoreductase that in turn furnishes  $\text{H}_2\text{O}_2$  for use by lactoperoxidase as an oxidizing agent. Thermal inactivation of these enzymes has been shown to result in greater oxidative stability in milk rich in linoleic acid (Hill, 1979).

Cytochromes have been reported at low concentrations in the milk fat globule membrane (Gregory *et al.*, 1976; Jarasch *et al.*, 1977). The powerful pro-oxidant properties of ferri-porphyrin proteins, together with their juxtaposition with lipids in the milk fat globule membrane, suggest that cytochromes may play a role in oxidation. Gregory *et al.* (1976) concluded that they do exert a role in milk lipid oxidation.

Clearly, more research is required to clarify the somewhat confused picture regarding the role of enzymes in the oxidation of milk lipids. However, the key factor affecting the susceptibility of milk to oxidation appears to be its relative content and distribution of pro-oxidants and antioxidants. Bruhn and Franke (1971) reported that spontaneous oxidation is directly proportional to the copper content and inversely proportional to the  $\alpha$ -tocopherol content of milk. Charmley *et al.* (1991) showed that intramuscular injection of cows with  $\alpha$ -tocopherol may overcome a spontaneous oxidized flavor problem caused by low levels of  $\alpha$ -tocopherol in milk. In general, milk from pasture-fed cows is less susceptible to oxidation due to a higher content of tocopherols than milk from cows given dry feed (Bruhn and Franke 1971; Urbach, 1989, 1990).

St. Laurent *et al.* (1990) investigated the effects on milk flavor of  $\alpha$ -tocopherol supplementation (0, 700 or 3000 IU/day) to a feed consisting of grain mix, hay and pasture in herds with a chronic spontaneous oxidized flavor problem.  $\alpha$ -Tocopherol supplementation resulted in improved milk flavor but no relationship was apparent between milk  $\alpha$ -tocopherol levels and the extent of flavor improvement. In this study, the flavor problem decreased significantly when the cows subsequently got access to spring pasture.

Barrefors *et al.* (1995) analyzed samples with and without oxidized flavor from two commercial herds. Their data indicated that oxidized milk samples had a higher linoleic acid content in the neutral fat fraction and contained a higher concentration of hexanal. At one of the farms, the concentration of both  $\alpha$ -tocopherol and  $\beta$ -carotene were lower in samples that developed off-flavor. They speculated that high-yielding cows fed high amounts of unsaturated fats in their feed needed higher dietary concentrations of  $\alpha$ -tocopherol and  $\beta$ -carotene.

#### **16.4. Factors that Affect the Oxidation of Lipids in Milk and Milk Products**

A range of environmental and physical factors, processing and storage conditions, and endogenous and exogenous chemical constituents and enzymes have been shown to influence the rate and extent of lipid oxidation in milk and

milk products. These include oxygen, light, endogenous and exogenous metals, antioxidants, ascorbic acid, tocopherols, carotenoids, thiols, proteins and enzymes, browning reaction products, milk fat globule membrane (MFGM) constituents, storage temperature and water activity.

The balance between pro-oxidant and antioxidant factors is critical for the oxidative stability of milk (Stapelfeldt *et al.*, 1999; Morales *et al.*, 2000). The degree of unsaturation of milk lipids is a factor influencing oxidation. Kristensen *et al.* (2004) obtained milk from cows fed a low-fat diet rich in cereals, which enhanced *de novo* fat synthesis and contained 21.3% unsaturated fatty acids and milk that contained 41.3% unsaturated fatty acids from cows fed a diet rich in soyabean oil. Buttermilk from the more unsaturated milk was less oxidatively stable during 11 days storage at 4°C than buttermilk from the more saturated milk as monitored by hydroperoxide and hexanal production. These workers also monitored during the storage period the levels of fat-soluble antioxidants ( $\alpha$ -tocopherol and  $\beta$ -carotene) and water-soluble antioxidants in the serum phase. The fat-soluble antioxidants were not consumed during storage of either of the two buttermilks. Interestingly, however, the antioxidative capacity of the serum phase decreased during storage with a similar time-course for the decrease in both types of buttermilk, suggesting that oxidation is initiated in the serum phase independently of fatty acid composition.

To a certain extent, the composition (including lipid composition) of cows' milk reflects feed composition (Bugaud *et al.*, 2001; Ramaswamy *et al.*, 2001). Certain feeding regimes have the potential to increase the level of polyunsaturated lipids in milk and the potential for oxidation (Barrefors *et al.*, 1995; Charmley and Nicholson, 1995; Hermansen, 1995; Focant *et al.*, 1998; Morales *et al.*, 2000; Bugaud *et al.*, 2001; Timmons *et al.*, 2001; Havemose *et al.*, 2004).

#### 16.4.1. Oxygen

Lipid oxidation, by definition, requires the presence of oxygen. However, the minimum residual oxygen concentration required for lipid oxidation in food products or in food packages may vary between different foodstuffs. Clearly, products with a large surface area or with a porous structure should theoretically be more predisposed to oxygen exposure and hence oxidation. Different packaging materials will influence oxygen transmission rate as will product to headspace ratio (Mortensen *et al.*, 2004). Transmission is also influenced by the partial pressure of oxygen inside and outside the packaging material, by storage temperature and by relative humidity (Robertson, 1993). Reducing the oxygen content in the package headspace will minimize oxidation rate (Hong *et al.*, 1995). However, a

low residual oxygen level of 0–5 ml/l headspace has been reported to result in rapid formation of oxidized off-flavor in Havarti cheese (Mortensen *et al.*, 2002). The same workers (Mortensen *et al.*, 2003) reported a saturation effect at approximately 1% residual oxygen for sliced Havarti cheese.

Oxygen has greater solubility in non-polar than in polar solvents and, hence, is more soluble in liquid milk fat than in whole raw milk. A substantial percentage of the total oxygen in whole milk exists in the fat phase, particularly at higher temperatures (Noll and Supplee, 1941; Timms *et al.*, 1982). Oxygen is excluded from the solid fat phase as it crystallizes. However, this may not imply a lower rate of oxidative deterioration at low temperatures. As the temperature of butter is reduced, oxygen excluded from the crystallized fat phase partitions into, and saturates, the liquid fat and aqueous phases of the butter. Thus, oxygen is available to react with the more unsaturated fat in the liquid phase and with phospholipids and pro-oxidants in MFGM fragments. The maximum rate of hydroperoxide production in irradiated butter is observed at  $-20^{\circ}\text{C}$ , which may reflect an increased rate of chain terminations at higher temperatures and reduced propagation reactions at lower temperatures (Hannan and Boag, 1952; Hannan and Shepherd, 1952).

Kinetically, ground-state (triplet- $^3\text{O}_2$ ) oxygen is not very reactive, which obviously restricts the oxidation of food lipids.  $^3\text{O}_2$  requires “activation” to facilitate oxidative reactions. Three principal processes are involved in the activation of oxygen (Fridovich, 1977):

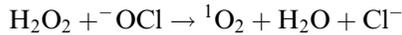
1. Photochemical excitation of an electron in  $^3\text{O}_2$  to a higher energy state may occur, thereby generating very reactive singlet oxygen,  $^1\text{O}_2$ . Photochemical excitation usually requires the intermediate participation of a photosensitizer (Ranby and Rabek, 1978), although other formation pathways exist. In food systems, the photosensitizer (e.g., riboflavin, chlorophyll, erythrosine) absorbs visible light and transfers energy to  $^3\text{O}_2$  to generate  $^1\text{O}_2$ .
2. Certain metals may interact with  $^3\text{O}_2$  to yield singlet-like  $\text{O}_2$  (Hanzlik, 1976).
3. Successive, univalent reduction of oxygen may yield reactive oxygen species. This reduction can be effected photochemically, chemically or enzymatically (Korycka-Dahl and Richardson, 1980). A number of enzymes, including Xanthine oxidoreductase in milk, are capable of producing large amounts of superoxide *via* the univalent reduction of oxygen (Fridovich, 1976).

In contrast to triplet oxygen, singlet oxygen is very electrophilic and readily reacts with unsaturated lipids with the formation of hydroperoxides,

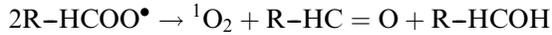
which can then decompose homolytically to initiate new free-radical chain reactions. Thus, initiation of oxidation by very low levels of  $^1\text{O}_2$  is sufficient to generate large numbers of reaction chains involving ground-state oxygen.

Korycka-Dahl and Richardson (1980) listed five possible ways by which  $^1\text{O}_2$  is generated in dairy products as a result of processing and storage:

1. Chemically, by reaction of residual hypochlorite with hydrogen peroxide:



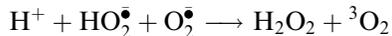
2. Chemically or enzymatically, by reactions involving metalloproteins:
  - (i) Protein -  $\text{M}^{n+} + ^3\text{O}_2 \rightarrow ^1\text{O}_2$ -like complex
  - (ii) Peroxidase -  $\text{OCl}^- + \text{H}_2\text{O}_2 \rightarrow ^1\text{O}_2$
3. Photochemically, *via* riboflavin or another sensitizer.
4. Self-reaction of secondary peroxy radicals:



5. Oxidation of superoxide by a restricted number of oxidizing agents can lead to  $^1\text{O}_2$ .

#### 16.4.1.1. Superoxide Radicals

The superoxide radical ( $\text{O}_2^\bullet$ ) is another reactive oxygen species. Although the superoxide radical may not react readily with unsaturated fatty acids, it is capable of oxidizing phenolic compounds such as tocopherols, thiols and ascorbic acid (Korycka-Dahl and Richardson, 1980). This, in turn, may lead to earlier oxidation of lipids. However, the significance of superoxide as a pro-oxidant in milk is unclear. Superoxide rapidly dismutates in water, yielding hydrogen peroxide which, itself, may be involved in oxidative reactions (Korycka-Dahl and Richardson, 1980):



This reaction is also catalyzed by superoxide dismutase (SOD), which occurs in milk at very low concentrations (Fox and Morrissey, 1981). Hydrogen peroxide can also be formed in milk as a result of microbial metabolism or by reduction of superoxide by ascorbic acid. A mean level of 0.02 mg/l hydrogen peroxide has been reported in milk (Toyoda *et al.*, 1982).

Superoxide may also univalently reduce hydrogen peroxide to yield the extremely reactive, electrophilic oxidant, hydroxyl radical (Schaich, 1980):



However, the significance of this reaction in foods is unclear.

Milk leucocytes have been shown to adhere to the MFGM (Peters and Trout, 1945a,b). The enzymatic generation of active oxygen species by phagocytic activity is another possible source of pro-oxidants (Salin and McCord, 1977; Kanner and Kinsella, 1983).

#### 16.4.1.2. Oxygen Removal

Removal of dissolved oxygen from liquid milk or its replacement by nitrogen reduces the intensity of oxidized flavor (Sharp *et al.*, 1941; Singleton *et al.*, 1963; Schroder, 1982). Available oxygen in the fat should be less than 0.8%, v/v, to prevent the production of a “tallowy” flavor in butteroil (Schaffer *et al.*, 1946). However, the production of all oxidized flavor is not reduced by lowering the concentration of dissolved oxygen. Schroder (1982) reported a reduction in light-induced, but not copper-induced, oxidized flavor development in oxygen-depleted milk. De-aeration to a very low oxygen level was necessary to prevent copper-induced oxidized flavor.

The oxidative stability of whole milk powder can be maintained for an extended period by vacuum treatment or replacement of oxygen with an inert gas (Greenbank *et al.*, 1946; Schaffer *et al.*, 1946). Tamsma *et al.* (1961) reported a statistically significant improvement in storage stability of whole milk powders packed in inert gases containing 0.1% oxygen compared with those packed at 1% oxygen level. Milk powders packed in the presence of glucose oxidase-catalase (oxygen scavenger system) and calcium oxide (dessicant) were comparable in flavor with samples stored in inert gas (Meyer and Jokay, 1960); the enzymes reduced the oxygen level to 0.5% in 1 week. Other workers have used various scavenging systems to deplete the oxygen level in stored milk powders with consequent improvement in keeping quality. One system utilized a mixture of 90% N<sub>2</sub> and 10% H<sub>2</sub> in the presence of palladium, which catalyzed the formation of water from the H<sub>2</sub> and residual oxygen to produce an almost oxygen-free atmosphere in the pack (Abbot and Waite, 1961). An oxygen-absorbing mixture (Na<sub>2</sub>SO<sub>3</sub> and CuSO<sub>4</sub>·5H<sub>2</sub>O) enclosed in porous paper pouches has also been shown to be effective (Jackson and Loo, 1959). Oxygen has been depleted to less than 0.001% within 24 h in packed milk powder using a scavenging system consisting of 95% N<sub>2</sub>, 5% H<sub>2</sub> and a platinum catalyst (Tamsma *et al.*, 1967).

Oxidized flavor is of minor importance in fermented dairy products such as cheese or yogurt (Wong *et al.*, 1973; Czulak *et al.*, 1974; Korycka-Dahl *et al.*, 1983). Several factors may be involved, including depletion of oxygen by the growth of starter bacteria, the acidic pH of the products, peptides produced by proteolysis, and the formation of antioxidants by microorganisms (Eriksson, 1982).

#### 16.4.2. Light

Many studies have shown that light is very effective in promoting off-flavor development in milk and milk products (Singleton *et al.*, 1963; Hedrick and Glass, 1975; Bray *et al.*, 1977; Sattar *et al.*, 1977a; Bradley, 1980; Nelson and Cathcart, 1984; Bartholomew and Ogden, 1990; Kim and Morr, 1996). Photooxidation of dairy products has been reviewed by Bradley (1980), Bosset *et al.* (1994, 1995), Skibsted (2000), Borle *et al.* (2001), and Mortensen *et al.* (2004). The extent of off-flavor development is a function of the wavelength involved, and the intensity and duration of exposure. Light has been shown to penetrate milk to an appreciable depth (Finley and Shipe, 1971; Newstead and Headifen, 1981).

The water-soluble vitamin, riboflavin, present in milk acts as a potent photosensitizer and has been implicated in the photooxidation of milk fat (Foote, 1976; Aurand *et al.*, 1977; Bekbolet, 1990). Aurand *et al.* (1977) suggested that singlet oxygen is involved based on the inhibitory effects of a singlet oxygen-trapping agent (1,3-diphenylisobenzofuran) or a singlet oxygen quencher (1,4-diazabicyclo-2,2,2-octane) on the oxidation of milk fat catalyzed by  $\text{Cu}^{2+}$ , enzymes or light. The ability of riboflavin to generate singlet oxygen in milk in its capacity as a photosensitizer has been confirmed by Bradley and Min (1992) and Berliner and Ogata (1997). After photodegradation, riboflavin breaks down to lumichrome and probably formylmethylflavin. Lumichrome is also a strong photosensitizer (Parks and Allen, 1977). Riboflavin has three absorption bands. The band with a maximum between 430–460 nm is the main band responsible for the photooxidation of food, especially milk and dairy products. Riboflavin transfers absorbed energy to other molecules such as dissolved oxygen in milk, thus generating reactive oxygen species. Sattar and Deman (1975) first demonstrated the correlation between duration of light exposure, presence of riboflavin and off-flavor development.

It has also been reported that riboflavin generates superoxide anion in milk exposed to fluorescent light and has been implicated in the destruction of other milk components, such as vitamin C, by light (Spikes and Livingstone, 1969; Korycha-Dahl and Richardson, 1979). The exposure of butter to light has been reported to result in the oxidation of cholesterol, giving rise

to 5-cholesten-3 $\beta$ ,7 $\alpha$ -diol and the 7 $\beta$ -epimer and, possibly, 6-cholesten-3 $\beta$ ,5 $\alpha$ -diol (Luby *et al.*, 1986a). Potential lipid-derived off-flavor in butter may be reduced by light-barrier packaging such as aluminum foil. Direct exposure to light is the principal factor affecting photooxidation of butter; temperature and duration of storage exert little effect on butter with sub-sensory levels of light-induced oxidation (Luby *et al.*, 1986b).

Light also influences milk flavor due to riboflavin-sensitized effects on milk proteins *via* oxidation of methionine to methional (3-methylthiopropionaldehyde) (Patton, 1954; Tada *et al.*, 1971; Sattar *et al.*, 1977b). Other amino acids, besides methionine, may be affected by the presence of light and riboflavin. Allen and Parks (1975) reported that exposure of milk serum to fluorescent light chemically modified 10 amino acids in immunoglobulins. Riboflavin-photosensitized oxidation of milk lipase, resulting in considerable loss of activity (80% loss in 30 min), has been reported in sunlight-exposed milk (Dimick, 1976). Light- and riboflavin-induced changes in cheese have also been reported (Deger and Ashoor, 1987).

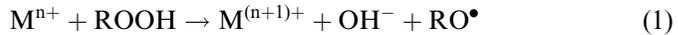
Recent work (Wold *et al.*, 2005) suggests that dairy products also have natural levels of porphyrins and chlorophylls and that these light-sensitive compounds play an important role in photooxidation of cheese. Fluorescent analysis indicated that photodegradation of porphyrins and chlorophylls correlated closely ( $R > 0.9$ ) with the sensory attribute of oxidized odor.

A number of studies have investigated the effect of packaging materials on the oxidative stability of stored liquid milk. Cladman *et al.* (1998) reported a significantly higher degree of lipid oxidation in milk packaged in high-density polyethylene (HDPE) jugs than in milk packaged in green polyethylene terephthalate (PET) bottles after 7 days of storage, presumably because the green PET reduced light exposure compared to the partly transparent HDPE. Erickson (1997) noted higher levels of lipid oxidation after 8 days storage in milk packaged in HDPE jugs compared to paper-board cartons. Zygoura *et al.* (2004) investigated the effect of packaging materials on the shelf-life of whole pasteurized milk. As expected, more lipid oxidation was observed over a 7-day storage period in clear PET than in pigmented packaging materials. Rysstad *et al.* (1998) reported that UHT milk stored at 6°C under 36 W vertical light was very oxidized in polyethylene containers, while milk stored under the same conditions was only slightly oxidized at 8 weeks in a non-foil paper-based carton and milk stored in aluminum foil cartons had no detectable oxidation. Hexanal production in cream powders stored for 35 weeks at 30°C was reported to be strongly influenced by exposure to fluorescent light and the presence of oxygen in the headspace (Andersson and Lingnert, 1998). Similarly, the intensity of oxidized flavor was greater in ice cream stored under fluorescent light than in the dark (Suttles and Marshall, 1993). Wold *et al.* (2002) used a solid sample

fluorescence technique to quantify the degree of light-induced oxidation in sour cream, goat cream cheese and Jarlsberg cheese and noted that changes in the fluorescence spectrum of light-exposed Jarlsberg were apparent 5–6 mm into the cheese. Few studies have investigated turnover times in fluorescent display cabinets. Haisman *et al.* (1992) reported turnover times of 2–3 days for liquid milk in such cabinets. Turnover times may be longer for plastic-packaged cheeses, which often have a large surface area exposed to light. Studies on Havarti cheese have indicated that an exposure times of < 12 h led to light-induced oxidative quality changes (Mortensen *et al.*, 2002).

### 16.4.3. Metals

Milk and milk products contain a wide variety of metal ions, including the pro-oxidant transition metal ions,  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ . Metal ions capable of undergoing reversible one-electron reductions are important pro-oxidants, which function primarily by decomposing hydroperoxides to generate new reaction chains (Labuza, 1971; Pokorny, 1987). Either the oxidized or reduced metal ion can decompose hydroperoxides to allow the following catalytic cycle to increase the rate of lipid oxidation:



Thus, a small quantity of an appropriate metal ion can generate large numbers of reaction chains by cycling between the oxidized and reduced forms. Although Fe and Cu in their reduced states are effective reducers of ROOH (Equation 1), they are inefficient oxidizers in their higher oxidation states (Equation 2). The oxidation step might be rate-limiting in the catalytic cycle unless alternative mechanisms are available for regenerating the reduced metal (Kochi, 1973). Constituents of foods that reduce  $\text{Fe}^{3+}$  (or  $\text{Cu}^{2+}$ ) may accelerate the breakdown of peroxides. Ascorbic acid, thiols and transient superoxide may provide reducing equivalents to accelerate these reactions. Interactions between metal ions and reducing groups (e.g., thiols) in milk are undoubtedly complex and may be responsible for inconsistencies and paradoxes in the literature (Yee and Shipe, 1982). Furthermore, ligands associated with transition metals can exert a profound influence on the catalytic properties of the bound metal (Cotton and Wilkinson, 1972; Hanzlik, 1976). The standard reduction potential for  $\text{Fe}^{3+}$  compared with that of  $\text{Cu}^{2+}$  suggests that  $\text{Fe}^{3+}$  is a much stronger oxidizing agent than  $\text{Cu}^{2+}$ . However, copper in milk is more pro-oxidant than iron (Haase and Dunkley, 1970; Jarrett, 1979; Rao and Murthy, 1987). This anomaly

probably reflects differences between the interactions of the two metals with other milk constituents (e.g., ascorbic acid, thiols, serine phosphate residues). The ligands associated with the metal ions also help to define their reactivities. The distribution of metals in milk and milk products is a complex function of relative solubility products and metal-ligand formation constraints which can, in turn, be influenced by processing, storage and seasonality.

Schwartz and Parks (1974) noted that awareness of the role of metal ions in the oxidation of milk fat has existed since 1905. It has long been recognized that Cu and Fe are the principal metals involved. Both these metals are normal constituents of milk but may also be present as contaminants: concentrations of Cu and Fe in U.S. milk have been reported to be highest in winter and lowest in summer (Murty *et al.*, 1972). Copper is present at a level of 20–400  $\mu\text{g}/\text{l}$  and Fe at a level of 100–900  $\mu\text{g}/\text{l}$  (Horvat *et al.*, 1965; Koops, 1969; Murty *et al.*, 1972; Johnson, 1974; Jarrett, 1979). However, as noted above, Cu is the principal catalytic metal in lipid oxidation.

The endogenous copper in milk is derived *via* the bloodstream from the cow's feed (Haase and Dunkley, 1970). It is unclear to what extent the copper content of the feed influences the copper content of milk (Mulder *et al.*, 1964; Riest *et al.*, 1967; Dunkley *et al.*, 1968a). However, the total level of endogenous copper in milk does not appear to be the key factor in spontaneous oxidation. King and Dunkley (1959b) and Samuelsson (1966) reported that oxidation may occur irrespective of copper content above a threshold value of 0.06  $\mu\text{g}/\text{kg}$ .

It has been shown that 10–35% of the endogenous copper and 20–47% of the endogenous iron in milk are associated with the MFGM (King *et al.*, 1959; Schwartz and Parks, 1974). However, only 2–3% of added copper and virtually no added iron become associated with the MFGM. While endogenous copper and iron in milk are complexed with proteins and are non-dialyzable at the normal pH of milk (King *et al.*, 1959), added copper and iron are dialyzable to some extent, suggesting that the interaction of added metals with proteins differs from the endogenous metals. It appears that the juxtaposition of a copper–protein complex with the phospholipids of the MFGM is an important factor in the development of oxidized flavor in liquid milk (Samuelsson, 1966).

Work has also been conducted on the removal of copper from milk. Thiosuccinylated aminoethyl cellulose has been used to remove more than 90% of the copper from milk (Roh *et al.*, 1976). Glass-bound trypsin has been used to inhibit metal-induced lipid oxidation (Shipe *et al.*, 1972). Further work by Gregory and Shipe (1975) showed that ageing milk before exposure to a metal catalyst reduced the extent of lipid oxidation and

enhanced the apparent anti-oxidative effect of trypsin treatment. The apparent mechanism involves trypsin hydrolysis of milk serum and MFGM proteins, which increases available sites for chelating metals, particularly  $\text{Cu}^{2+}$ , into non-pro-oxidant complexes.

Enrichment of whole milk before pasteurization with ferrous iron has been reported to give rise to oxidized flavor (Edmondson *et al.*, 1971). Aeration before addition of the iron reduced the effect. Kurtz *et al.* (1973), however, reported that milk powder can be fortified with iron in amounts equivalent to 20 mg/l of iron in reconstituted skim milk without development of oxidized flavor.

## 16.5. Antioxidants

Synthetic antioxidants are used widely in food products to inhibit the progress of lipid oxidation. However, their use in dairy products is prohibited in most countries. Experimental studies of the efficacy of antioxidants such as butylated hydroxy anisole (BHA), hydroxyquinone, dihydroquercetin and gallic acid esters in dairy products have been conducted (Sidhu *et al.*, 1975, 1976). Studies on the use of antioxidants in dairy products show that their effectiveness varies in different products. While norhydroguaiaretic acid inhibits the development of oxidized flavor in liquid milk, it promotes autoxidation in milk fat (Hammond, 1970). Tocopherols are very effective inhibitors of spontaneous or copper-induced oxidation in liquid milk (Dunkley *et al.*, 1967; King, 1968) but have little effect in whole milk powder (Abbot and Waite, 1965). Other antioxidants that have been shown to exert protective effects are dodecyl gallate in spray-dried whole milk (Abbot and Waite, 1962), ascorbyl palmitate in lactic butter (Koops, 1964) and propyl gallate and quercetin in butteroil (Wyatt and Day, 1965). Anhydrous bovine or buffalo milk fats (ghee) may be stabilized when stored in a hot climate by combinations of phenolic antioxidants (BHA, butylated hydroxy toluene (BHT), propyl gallate (PG)) and ascorbic acid (Helal *et al.*, 1976).

Wade *et al.* (1986) reported that BHA and BHT were effective in retarding oxidation of anhydrous milk fat but DL- $\alpha$ -tocopherol acted as a pro-oxidant. Natural antioxidants in betel and curry leaves have also been reported to retard oxidation of anhydrous milk fat (Sharma, 1981; Parmer and Sharma, 1986). Amr (1991) reported that turmeric and wheat grits were as effective as BHA and BHT in controlling oxidative rancidity in sheep's anhydrous milk fat for up to 4 months. However, rosemary, sage, rue and fennel exerted pro-oxidant effects. Quercetin and rutin are reported to be efficient antioxidants in butter (Eriksson, 1987).

Metal-chelating agents, such as citric acid and phosphoric acid, act as synergists in conjunction with antioxidants (Badings, 1960). Salts of ethylenediaminetetraacetic acid (EDTA) have been shown to inhibit oxidation in this manner (Arrington and Krienke, 1954; King and Dunkley, 1959a).

### 16.5.1. Ascorbic Acid

Ascorbic acid is a very effective scavenger of alkoxy radicals and hence is an effective antioxidant (Niki, 1991; Frankel, 1998). However, under certain circumstances, ascorbic acid exerts a pro-oxidant effect. Andersson and Oste (1994) reported that ascorbic acid concentration in unpasteurized milk was highest in March or August (20–27 mg/l) and lowest in October (12 mg/l). Vitamin C level typically decreases during storage and following heating of milk (Korhonen and Korpela, 1994). Concentrations of ascorbic acid above those in normal milk (approximately 20 mg/l) provide antioxidant protection; however, at the concentrations in milk, ascorbic acid acts as a pro-oxidant. Olson and Brown (1942) showed that ascorbic acid was crucial to the development of oxidized flavor in cream. They reported that cream washed free of ascorbic acid did not develop oxidized flavor when contaminated with copper and stored for 3 days. They postulated that ascorbic acid reduces  $\text{Cu}^{2+}$  to  $\text{Cu}^+$ , which in turn reduces molecular oxygen to hydrogen peroxide that oxidizes lipids in the MFGM. Pont (1952) reported that addition of ascorbic acid to washed cream, even in the absence of added copper, promoted the development of an oxidized flavor. Krukovsky and Guthrie (1945) and Krukovsky (1955, 1961) showed that added copper did not promote oxidation in milk or butter depleted of ascorbic acid and that oxidation of ascorbic acid-free milk could be initiated by addition of ascorbic acid. In a series of papers (King and Dunkley, 1959a; Smith and Dunkley, 1962a,b,c; Haase and Dunkley, 1969a,b,c) that involved addition of copper ions to milk and model systems, judicious application of specific chelators for copper ions, addition or specific destruction of ascorbic acid in milk and model systems, and the use of reducing agents other than ascorbic acid, the primary function of copper and ascorbic acid in catalyzing lipid oxidation was carefully documented. A specific association between copper and ascorbic acid as the ultimate pro-oxidant was proposed.

However, while some reports (Schwartz and Parks, 1974) indicate a correlation between the oxidation of ascorbic acid and the development of an oxidized lipid flavor, Smith and Dunkley (1962c) concluded that the oxidation of ascorbic acid alone cannot be used as an index of lipid oxidation. They reported that although ascorbic acid oxidation curves for homogenized and pasteurized milk were similar, the homogenized samples had a significantly lower tendency to develop oxidized flavor.

Several workers have shown that a high concentration of ascorbic acid added to liquid milk inhibits oxidation. Chilson (1935) suggested that added ascorbic acid acts as a reducing agent, which is oxidized more readily than milk fat. Bell *et al.* (1962) suggested that addition of L-ascorbic acid to cream produced a medium less conducive to oxidation by lowering the oxidation-reduction potential. Addition of an adequate level of surface-active ascorbyl palmitate to milk products may retard lipid oxidation by orientation at the lipid-aqueous interface where it intercepts free radicals (Badings and Neeter, 1980).

### 16.5.2. Tocopherols

Vitamin E consists of eight vitamers of which  $\alpha$ -tocopherol is the principal one in bovine milk (Lindmark-Mansson and Akesson, 2000).  $\alpha$ -Tocopherol acts as a free-radical scavenger. The tocopheryloxy radical formed is relatively stable and can be reconverted to tocopherol by reduction with ascorbic acid. Tocopherols generally act as antioxidants in lipids (Kamaleldin and Appelqvist, 1996). At high concentrations, they may exert a pro-oxidant effect (Hamzawi, 1990), but this is highly unlikely to occur in milk. Milk fat contains approximately 20  $\mu\text{g}$   $\alpha$ -tocopherol/g (Erickson and Dunkley, 1964; Kanno *et al.*, 1968; Bruhn and Franke, 1971; Jensen, 1995). Tocopherol concentrations are at least three-fold higher in lipids of the MFGM than in the core of the fat globule (Erickson *et al.*, 1963). During storage of cream containing added  $\text{Cu}^{2+}$  and ascorbic acid, total destruction of tocopherols in the MFGM was observed compared with 30% destruction in the butteroil due to the proximity of tocopherols in the MFGM to pro-oxidants and highly oxidizable phospholipids.

The principal factor that influences the  $\alpha$ -tocopherol content of milk is the feed of the cow, as influenced by the season of the year. Kanno *et al.* (1968) reported that summer milk produced on green pasture feed averaged 33.8  $\mu\text{g}$   $\alpha$ -tocopherol/g fat, while winter milk produced on dry-lot feeding averaged 21.6  $\mu\text{g}$   $\alpha$ -tocopherol/g fat. Similar findings have been reported by King *et al.* (1967) and Seerless and Armstrong (1970).

The feasibility of increasing the  $\alpha$ -tocopherol concentration of milk by supplementation of the feed has been investigated in many studies (Dunkley *et al.*, 1966, 1967; King *et al.*, 1966; St. Laurent *et al.*, 1990; Barrefors *et al.*, 1995; Focant *et al.*, 1998; Granelli *et al.*, 1998). These studies showed that when feed was supplemented with varying levels of  $\alpha$ -tocopheryl acetate, the  $\alpha$ -tocopherol content of the milk was increased with consequent increased resistance to spontaneous and copper-induced oxidation. King *et al.* (1967) reported that when feed was supplemented to achieve an intake of 1 g  $\alpha$ -tocopherol per day per cow, oxidation was effectively controlled in milk

contaminated with 0.1  $\mu\text{g}/\text{kg}$  copper. However, other studies have shown no beneficial effect of supplementing the diet with vitamin E (Schingoethe *et al.*, 1979; Charmley and Nicholson, 1995).

Only about 2% of ingested  $\alpha$ -tocopherol is actually transferred to the milk (King *et al.*, 1966; Dunkley *et al.*, 1968b; Schingoethe *et al.*, 1979). Consequently, the economics of direct supplementation of feed with  $\alpha$ -tocopherol are unfavourable (Bruhn *et al.*, 1976). If protected supplements are fed, however, the potential for transfer to milk is much greater. Goering *et al.* (1976) fed protected safflower supplement to cows and reported a 200% increase in the  $\alpha$ -tocopherol content of the milk. Control of oxidized flavor by direct addition of emulsified  $\alpha$ -tocopherol to milk can be achieved with only 1% of the amount required by ration supplementation (Weihsrauch, 1988).

A significant correlation exists between the  $\alpha$ -tocopherol content of milk fat and oxidative stability (Krukovsky *et al.*, 1950). Tocopherol concentration in MFGM lipids shows a closer correlation to oxidative stability than the tocopherol content of butteroil (Erickson *et al.*, 1963). A direct relationship has been observed between tocopherol concentration and the level of copper that can be tolerated by milk (King *et al.*, 1966).

Tocopherols have been reported to act as free-radical scavengers (Terao *et al.*, 1980) but have also been shown to quench  $^1\text{O}_2$  via a charge-transfer quenching mechanism (Yamauchi and Matsushita, 1977; Burton and Ingold, 1981). The ratio of  $^1\text{O}_2$  quenching rates of  $\alpha$ -,  $\gamma$ - and  $\delta$ -tocopherols were found to be 100:69:38. Each tocopherol molecule can deactivate about 120 molecules of  $^1\text{O}_2$  before it is destroyed (Zweig and Henderson, 1975).

### 16.5.3. Carotenoids

Molecules containing conjugated double bonds are oxidized more rapidly than those with the same number of nonconjugated double bonds. Oxidation of  $\beta$ -carotene is very complex and may take several routes. Generally, conjugated double-bond systems favor radical addition rather than abstraction reactions (Scott, 1965). Addition of a methyl radical to conjugated double bonds is very rapid compared with abstraction reactions (Pryor *et al.*, 1972). Carotenoids undergo radical addition reactions leading to bleaching and a variety of compounds characteristic of lipid oxidation in general (Teixeira Neto *et al.*, 1981). However, very electrophilic oxidizing free radicals may abstract an electron from  $\beta$ -carotene to yield a radical  $\beta$ -carotene cation. Epoxides are readily formed at the double bond in the  $\beta$ -ionone ring and such epoxidized carotenoids are commonly found in naturally-occurring pigments (McCormick *et al.*, 1978). Novel oxidation products containing the  $\beta$ -ionone moiety have been implicated in off-flavor

development in dairy products. Oxidation of vitamin A should follow the same patterns as  $\beta$ -carotene. Although the conjugated diene in vitamin D reacts readily with photogenerated  $^1\text{O}_2$  to form endoperoxides, little is known about the oxidation of vitamins D and K in milk and milk products.

The importance of  $^1\text{O}_2$  as an initiator of oxidation has increased interest in the prevention of singlet oxygen reactions by quenching to ground-state oxygen ( $^3\text{O}_2$ ). Food constituents such as carotenoids, tocopherols and ascorbic acid have been reported to exert this effect (Carlsson *et al.*, 1976; Krinsky, 1979; Matsushita and Terao, 1980; Fakourelis *et al.*, 1987; Warner and Frakel, 1987; Jung and Min, 1991; Mortensen *et al.*, 2001). Quenchers must either be capable of accepting energy from the  $^1\text{O}_2$  molecule that lies 22.4 kcal above the ground state (energy-transfer quenching) or have the ability to donate electrons to  $^1\text{O}_2$  (charge-transfer quenching). Quenching of  $^1\text{O}_2$  by  $\beta$ -carotene is an example of energy-transfer quenching, whereas tocopherols, amines and phenols have been shown to exert a charge-transfer quenching mechanism (Bradley and Min, 1992).

Energy transfer from  $^1\text{O}_2$  to  $\beta$ -carotene leads to the formation of ground-state oxygen and an excited triplet-state quencher (Seeley and Meyer, 1971; Foote *et al.*, 1974).  $\beta$ -Carotene is known to be one of the most potent quenchers of  $^1\text{O}_2$ , with one molecule estimated to quench 250–1000 molecules of  $^1\text{O}_2$  (Foote and Denny, 1968; Foote, 1976). The rate of quenching is influenced by the number of conjugated double bonds present. Carotenoids with nine or more conjugated double bonds are efficient quenchers, whereas those with seven or less are not capable of accepting energy from  $^1\text{O}_2$ .  $\beta$ -Carotene has been shown to inhibit chlorophyll-sensitized photo-oxidation of methyl linoleate (Terao *et al.*, 1980). Using a model dairy spread (water-in-oil emulsion), Hansen and Skibsted (2000) showed that  $\beta$ -carotene provided good protection against light-induced lipid oxidation and riboflavin degradation. The protection of the riboflavin was due to the competing absorption of the light by the carotene. Outside the absorption band of the carotene (i.e., <366 nm), this protective effect disappeared. This indicated that the protective mechanism of the carotene in this case was an absorption (filter) effect of the incident light and not a quenching effect of radical or singlet oxygen.

#### 16.5.4. Thiols

Pasteurization of milk increases its susceptibility to spontaneous (Bergman *et al.*, 1962), copper-induced (Smith and Dunkley, 1962a) and photo-induced (Finley, 1968) oxidation. Postulated explanations generally implicate migration of copper to the cream phase of milk (Sargent and Stine, 1964).

However, an inhibitory effect of high heat treatment on the oxidative deterioration of milk and milk products has been reported by many investigators who have attributed this effect to the activation of thiol groups (Josephson and Doan, 1939; Tamsma *et al.*, 1962; Wilson and Herreid, 1969; Schwartz and Parks, 1974; Baldwin and Ackland, 1991; Saidi and Warthesen, 1995; Tong *et al.*, 2000). The principal sources of thiols in milk are the fat globule membrane (McPherson and Kitchen, 1983) and the serum proteins, particularly  $\beta$ -lactoglobulin (Larsson and Jenness, 1950; Schwartz and Parks, 1974).

While, historically, thiols resulting from heat treatment of milk have been considered as performing an antioxidant function in milk as univalent reducing agents, peroxide decomposers or as metal ligands, it is also possible that they may exert a pro-oxidant role. Univalent autoxidation of thiol yields thiyl radicals, superoxide and hydrogen peroxide may provide a basis for a pro-oxidant role (Yee and Shipe, 1982). These workers proposed that copper-catalyzed oxidation of thiol groups may generate pro-oxidants in milk. Autoxidation of thiols is known to generate substantial amounts of superoxide anion (Misra, 1974). Yee and Shipe (1982) concluded that thiol groups in milk may be pro-oxidant or antioxidant, depending upon the conditions. Free thiol groups in the presence of copper promoted the oxidation of emulsified methyl linoleate in their model system, whereas free thiol groups in the presence of haem behaved as antioxidants.

Sulphydryl oxidase, an indigenous milk enzyme, has been proposed for the oxidation of thiols in UHT milk to reduce cooked flavor and also thereby to serve as an antioxidant, in conjunction with lactoperoxidase (to destroy the resultant  $H_2O_2$ ), by obviating pro-oxidants resulting from autoxidation of thiols (Swaisgood and Abraham, 1980).

Stapelfeldt *et al.* (1997a) determined the oxidative stability of high-heat, medium-heat and low-heat whole milk powder under different storage conditions. The sensory quality dropped to an unacceptable level for low-heat powder after 33 days and was paralleled by a decrease of "free" thiol groups to an unmeasurable level. In contrast, medium-heat and high-heat powders retained good sensory quality and the initial level of free thiol groups was reduced by only one-third after 63 days of storage.

### 16.5.5. Proteins and Enzymes

Caseins possess significant antioxidant activity, which may be related, in part, to their hydrophobic nature (El-Negoumy, 1965; Taylor and Richardson, 1980b; Allen and Wrieden, 1982a; Ericksson, 1982) and orientation of potential antioxidant side-chains of constituent amino acids at the lipid interface. Brunner (1974) reported retardation of lipid oxidation in

homogenized milk when milk fat globules are resurfaced with casein. Caseins can also bind metals to phosphoserine residues (Manson and Cannon 1978; Hegenauer *et al.*, 1979a,b). Auklakh and Stine (1971) reported that sodium  $\alpha_{s1}$ - and  $\beta$ -caseinates bound 2 mol  $\text{Cu}^{2+}$  per mol protein. The association of peroxidizing lipids with proteins can be especially damaging to the physicochemical properties of the protein (Schaich, 1980). The major whey proteins are considerably less effective as antioxidants than the caseins (Taylor and Richardson, 1980b; Allen and Wrieden, 1982a). Lactoferrin has been shown to inhibit peroxidation induced by  $\text{Fe}^{2+}$ , presumably by binding  $\text{Fe}^{2+}$  (Gutteridge *et al.*, 1981; Allen and Wrieden, 1982b). Binding of iron to lactoferrin may decrease the conversion of hydrogen peroxide into hydroxyl radical *via* the Fenton-type reaction (Lindmark-Mansson and Akesson, 2000). Bovine lactoferrin has been reported to inhibit oxidation of ascorbic acid and tryptophan (Bihel and Birlouez-Aragon, 1998).

Lactoperoxidase was strongly pro-oxidant in the presence or absence of added  $\text{Cu}^{2+}$  or  $\text{Fe}^{3+}$  in a trilinolein emulsion model (Allen and Wrieden, 1982a) or in high linoleate milk (Hill, 1979). Pasteurization at  $72^\circ\text{C}/15\text{--}20$  s had little effect, but following heating at  $80^\circ\text{C}$  for  $15\text{--}20$  s, lipid oxidation was greatly reduced. Hill (1979) and Allen and Wrieden (1982a) also showed that superoxide dismutase and catalase exert a strong antioxidant effect when added in their model systems. However, addition of  $\text{Cu}^{2+}$  ( $10\ \mu\text{M}$ ) with superoxide dismutase to the emulsion was pro-oxidant and might compete with the enzyme for  $\text{O}_2^\bullet$  to convert it to pro-oxidant species such as  $\text{OH}^\bullet$  (Allen and Wrieden, 1982b). Superoxide dismutase has been detected in and isolated from milk (Hill, 1975; Asada, 1976; Korycka-Dahl *et al.*, 1979), but it is apparently present at insufficient levels to provide substantial antioxidant protection (Holbrook and Hicks, 1978; Fox and Morrissey, 1981). However, the observed inhibition by superoxide dismutase of lipid oxidation catalyzed by Xanthine oxidoreductase (Holbrook and Hicks, 1978) may have confounded the hypothesis of Smith and Dunkley (1960) on the importance of Xanthine oxidoreductase in spontaneous oxidation of milk lipids (Section 8.3.1). However, Holbrook and Hicks (1978) were unable to correlate spontaneous oxidation of milk with its content of superoxide dismutase.

Amino acids have been reported to act as antioxidants, pro-oxidants and/or to have no effect on lipid oxidation (Farag *et al.*, 1978; Taylor and Richardson, 1980a). Antioxidant effects have been attributed to: (1) their primary functional groups; (2) chelation of pro-oxidant metals; (3) regeneration of primary antioxidants; or (4) synergism with other food constituents (Chen and Nawar, 1991b). Faraget *et al.* (1978) suggested that protonated amino groups accelerate lipid oxidation while non-protonated amino groups

inhibit oxidation. The antioxidant/pro-oxidant effects of amino acids in emulsion systems may be pH dependent (Riisom *et al.*, 1980). Pro-oxidant activity is enhanced at lower pH.

Chen and Nawar (1991b) examined the effects of amino acids and amino acid analogues on fat oxidation in milk. All the amino acids tested (cysteine, tryptophan, lysine, alanine, serine, histidine and tyrosine) significantly prolonged the induction period of lipid oxidation, with cysteine, tryptophan and lysine showing the most pronounced effects. Comparison of the effects of amino acids with structurally similar analogues, in which the amino group was absent or blocked, indicated that the primary amino group plays a major role in the inhibitory activity of amino acids. An amino group on the side chain of an amino acid also exerted an antioxidant effect, although to a lesser degree than that of  $\alpha$ -amino groups. The indolyl group of L-tryptophan also exerted a strong antioxidant effect.

### 16.5.6. Products of Browning Reactions

Carbonyl-amine reactions, such as those between lactose and milk proteins, have been reported to produce potent antioxidants (Dugan, 1980; Eichner, 1980; Ericksson, 1982). Browning reaction products can stabilize milk fat considerably (Wyatt and Day, 1965). However, it is important to note that browning reaction products may exert adverse nutritional and toxicological effects (O'Brien and Morrissey, 1989).

The effect of browning compounds on the auto-oxidative stability of ghee has been reported by Nath and Murthy (1988). Browning compounds were prepared by heating leucine and dicarbonyls (either dihydroxyacetone, methyl glyoxal or glyoxal). All three types of browning oil, when added, afforded protection against autoxidation of ghee heated to 120°C for 5 min. However, BHT (0.02%) was a more powerful antioxidant than the most effective dicarbonyl/amino acid combination (dihydroxyacetone/leucine).

Lingnert *et al.* (1983) reported that addition of 0.3% of the Maillard products of histidine and glucose to milk powder and storage under nitrogen gave the reconstituted milk both a low initial intensity of "cardboard" flavor and almost total inhibition of its further development after reconstitution.

Calligaris *et al.* (2004) studied changes in antioxidant and pro-oxidant activity in milk subjected to different heat treatments. Their results indicated that short heat treatments can be potentially responsible for a depletion in the overall antioxidant properties of milk. Only the application of severe heat treatments, associated with the formation of brown melanoidins, allowed a recovery and even a possible increase in the antioxidant properties of milk.

## 16.6. Milk Fat Globule Membrane (MFGM)

Fat in milk exists primarily in the form of globules surrounded by a complex membrane, which contains a mixture of unsaturated phospholipids, proteins, glycoproteins and other minor components (Mulder and Walstra, 1974; Keenan *et al.*, 1983; see Chapter 4; Keenan and Dylewski, 1995). The proximity of unsaturated phospholipids to various pro-oxidants in the lipoprotein matrix makes the MFGM a focal point for the oxidation of milk lipids (Mulder and Walstra, 1974; Bouzas *et al.*, 1985). O'Mahony and Shipe (1970) found that the concentration of phosphatidylethanolamine (PE) was lowest in milk classified as least susceptible to copper-induced oxidation. PE is known to bind  $\text{Cu}^{2+}$  strongly (Morita and Fujimaki, 1972). Reconstituted milk was reported to be more stable to oxidation than regular milk due to removal of membrane material (Krukovsky, 1952). The rate of copper-catalyzed oxidation of cream containing MFGM was faster than when the membrane was removed. Ascorbic acid (10 mg/kg) accelerated the oxidation of cream under these conditions (Chen and Nawar, 1991a). A decrease in the relative concentration of membrane phospholipids and copper was proposed as the mechanism by which the development of oxidized and "tallowy" flavor in homogenized milk was inhibited (Tarassuk and Koops, 1960). Rapid oxidation of isolated MFGM was demonstrated by King (1962, 1963). Oxidation in isolated MFGM was influenced by Cu and ascorbic acid concentration. Once oxidation is initiated in the MFGM, diffusion of the propagating chain reaction radicals into the more saturated fat globule core from the fat-plasma interface results in generalized oxidation of milk fat triglycerides.

About one-third of the phospholipids in freshly drawn milk are located in the milk serum as small lipoprotein particles, sometimes referred to as "milk microsomes." Their proportion in milk serum can be increased in processed milk as a result of disruption of the MFGM and release of membrane phospholipids into the aqueous phase (Mulder and Walstra, 1974; McPherson and Kitchen, 1983). Modification of the MFGM by processing treatments that may alter the distribution of pro-oxidants and antioxidants can markedly affect the stability of milk (McPherson and Kitchen, 1983).

Xanthine oxidoreductase, a metalloprotein abundant in the MFGM, may also be partially responsible for the susceptibility of the membrane to lipid oxidation (Allen and Humphries, 1977; Aurand *et al.*, 1977; Bruder *et al.*, 1982; Bouzas *et al.*, 1985). Allen and Humphries (1977) prepared two protein fractions from MFGM and found that oxidative activity resided almost entirely in the first fraction, devoid of phospholipids, but richer in Xanthine oxidoreductase. They proposed that the metalloprotein, and not

phospholipids, was probably responsible for the inherent oxidative capability of the membrane material.

The mechanism(s) by which Xanthine oxidoreductase exerts its pro-oxidant effect(s) is not fully understood. Hydrogen peroxide, resulting from oxidation of a suitable substrate by Xanthine oxidoreductase, could oxidize milk lipids. However, normal milk contains little or no substrate for the enzyme. A possible mechanism involving interaction between native and denatured Xanthine oxidoreductase in MFGM and lactoperoxidase or copper in milk serum has been proposed (Hill, 1979; Allen and Wrieden, 1982b).

Hill (1979) used milk containing up to 35% (w/w) linoleic acid in the milk fat to investigate the mechanism of action of Xanthine oxidoreductase. He proposed a coupled enzyme system in which lactoperoxidase catalyses lipid oxidation, generating aldehyde substrates for Xanthine oxidoreductase that in turn generates  $H_2O_2$  for use by lactoperoxidase as an oxidizing agent. Hill (1979) reported that milk rich in linoleic acid was much more oxidatively stable when pasteurized at  $80^\circ C/15$  s than after pasteurization at  $72^\circ C/15$  s. The increased stability was attributed to thermal inactivation of the oxidative enzymes at the higher temperature since after pasteurization of the milk at  $80^\circ C/15$  s, rapid development of oxidized flavor occurred if Xanthine oxidoreductase or lactoperoxidase was added. When substrate for Xanthine oxidoreductase was added also, oxidative processes were accelerated. Addition of small amounts (1 mg/l) of superoxidase dismutase and catalase improved the oxidative stability of this milk, indicating that  $O_2^\bullet$  is involved in the oxidative process. However, oxidation of milk lipids after addition of 0.1 mg/kg  $Cu^{2+}$  was not inhibited by superoxide dismutase and catalase. When formate (an  $\bullet OH$  scavenger) was added to the milk, lipid oxidation was inhibited, suggesting that  $\bullet OH$  was the active pro-oxidant. Hill (1979) postulated that two major systems in milk catalyze lipid oxidation:

1. Generation of  $\bullet OH$  by copper-ascorbic acid;
2. Generation of  $O_2^\bullet$  and  $^1O_2$  by Xanthine oxidoreductase-lactoperoxidase.

Allen and Wrieden (1982b) used a trilinolein model system to confirm the strong pro-oxidant role of lactoperoxidase which was retarded by heating at  $80^\circ C/20$  s. They proposed that, in addition to its enzymatic effects, lactoperoxidase was pro-oxidant by virtue of generalized haem catalysis. In the presence of  $10 \mu M$  added  $Cu^{2+}$ , Xanthine oxidoreductase rapidly oxidized trilinolein in the absence of a substrate for Xanthine oxidoreductase (Allen and Wrieden, 1982b). They suggested that the added  $Cu^{2+}$  inactivated the Xanthine oxidoreductase and that any  $O_2^\bullet$  produced could be converted to very strong oxidizing species by the  $Cu^{2+}$  bound to the inactive enzyme. The FAD associated with Xanthine oxidoreductase may also act as a photosensitizer in the production of  $O_2^\bullet$  (Korycka-Dahl and Richardson, 1978). The potentially longer lifetime of

$O_2^{\bullet}$  in the membrane lipids in association with bound  $Cu^{2+}$  could make these reactions of some significance in lipid oxidation.

Several workers have reported low concentrations of cytochromes in the MFGM (Baillie and Morton, 1958a,b; Plantz *et al.*, 1973; Gregory *et al.*, 1976; Bernstein, 1977; Jarasch *et al.*, 1977). Ferri-porphyrin proteins have been shown to be powerful pro-oxidants (Kendrick and Watts, 1969). Furthermore, their proximity to unsaturated phospholipids in the MFGM and the longer life-times of active oxygen species in a nonpolar environment, suggest a role for cytochromes in the oxidation of MFGM lipids. Bernstein (1977) reported a  $b_5$ -type cytochrome and a carbon monoxide-binding cytochrome in MFGM. The CO-binding cytochrome promoted lipid oxidation to the same extent as hemoglobin, whereas the native  $b_5$ -type cytochrome was inactive. Thermal processing of milk may expose or mask ferri-porphyrin groups in MFGM and, hence, may influence lipid oxidation.

## 16.7. Storage Temperature

The effect of storage temperature on the oxidative stability of milk and milk products is unclear. Storage, in air, at 2°C inhibited the development of oxidized flavor in dry whole milk when compared with control samples held at 38°C (Pyenson and Tracy, 1946). Oxidative deterioration of UHT cream occurred two to three times more rapidly at 18°C than at 10°C, while little or no oxidation occurred at 4°C (Downey, 1969). The oxidation–reduction potential of butter and the rate of flavor deterioration have been reported to increase as the storage temperature increased (Weihrach, 1988).

In a study on butteroil held at a temperature ranging from –10 to +50°C, oxidation rate increased with increasing temperature but the same flavor was formed on storage and the reaction sequence for flavor formation was similar at all temperatures (Hamm *et al.*, 1968). Dunkley and Franke (1967) reported a decrease in flavor intensity and thiobarbituric acid (TBA) values in liquid milk as storage temperature was increased from 0 to 4 to 8°C. Schwartz and Parks (1974) reported that condensed milk stored at –17°C was more susceptible to oxidized flavor development than at –7°C.

Kristensen (2001) reported that increasing the storage temperature from 5 to 37°C for processed cheese resulted in significantly enhanced oxidation which was apparent after a few days of light-exposed storage.

## 16.8. Water Activity

Labuza (1971) has described the complex relationship between water activity ( $a_w$ ) and lipid oxidation, with a minimum observed at intermediate  $a_w$  (~0.4)

levels and an increased rate of oxidation at either very low or high  $a_w$ . At an  $a_w$  values  $>0.8$ , the rate of lipid oxidation decreases. Labuza (1971), Karel (1980) and Schaich (1980) explained these complex relationships, suggesting that at very low  $a_w$ , lipid oxidation is favored because a water monolayer is not available to mask pro-oxidants or to retard the decomposition of hydroperoxides by hydrogen bonding. As the amount of water is increased to form a monolayer, pro-oxidants may be masked *via* hydration and the monolayer serves as a barrier to oxidation. Higher  $a_w$  promotes lipid oxidation by mobilizing pro-oxidants and facilitating their diffusion through the food. However, very high  $a_w$  values retard oxidation by diluting the reactants.

In contrast to the hypothesis of Labuza (1971), Loncin *et al.* (1968) reported that autoxidation of milk powder was stimulated by water activity below 0.11 and unaffected by water activities between this value and 0.75. Stapelfeldt *et al.* (1977a) investigated the oxidative stability of whole milk powder at water activities of 0.11, 0.23 and 0.33 at 25°C and 0.11, 0.17 and 0.31 at 45°C for 2 months of storage. As one would expect, lipid oxidation was affected greatly by higher storage temperature. In contrast to the generalization of Labuza (1971), least oxidation was observed at a water activity between 0.11 and 0.23 and most oxidation at higher water activities (0.31 at 45°C).

## 16.9. Measurement of Lipid Oxidation

Routine procedures to assay the extent of oxidation in lipids and lipid-containing foods should be simple, reliable and sensitive. Results from routine procedures should ideally correlate well with results obtained from sensory taste panels. St. Angelo (1996) has described volatile compound profiles formed during lipid oxidation in different groups of food products. However, because of the complexity of lipid oxidation, no single test can be equally useful at all stages of the oxidative process. The methods should be capable of detecting autoxidation before the onset of off-flavor. This is particularly true in the case of milk products where a low level of oxidation can lead to off-flavor.

Measurement of hydroperoxides is the classical method for quantifying lipid oxidation and a variety of assay procedures are available. The oxidation of ferrous to ferric iron by hydroperoxides in the presence of ammonium thiocyanate to produce ferric thiocyanate, which can be quantified spectrophotometrically at 505 nm, has been used extensively to study lipid oxidation in milk (Loftus-Hills and Thiel, 1946). Newstead and Headifen (1981) recommend that extraction of fat from whole milk powder be carried out in the dark when using this procedure to avoid artefactually high

peroxide values. A second assay procedure for hydroperoxides is based on the reaction of oxidized fat with 1,5-diphenyl-carbohydrazide to yield a red-colored product (Hamm *et al.*, 1965). A third procedure is based upon the liberation of iodine from potassium iodide by hydroperoxides (AOCS, 1971). A standardized protocol exists for peroxide value determination in butterfat (IDF, 1991). A major caveat with procedures based on the direct or indirect determination of hydroperoxides is that they may not correlate well with the level of off-flavor in the product, particularly when the oxidative process is at an advanced stage (Kliman *et al.*, 1962). During the course of oxidation, peroxide values reach a peak and then decline. However, the early stage of lipid oxidation in dairy products can be followed by HPLC analysis of hydroperoxides or the peroxide value (Emmons *et al.*, 1986).

Procedures involving thiobarbituric acid (TBA) as an analytical reagent have also been used widely to follow the progress of autoxidation in dairy products (Dunkley and Jennings, 1951; King, 1962). Both procedures are based on the condensation of two molecules of TBA with one molecule of the oxidation end-product, malonaldehyde, resulting in the formation of a red-colored complex which can be determined spectrophotometrically at 532 nm. However, compounds other than malonaldehyde may react with this reagent to give artefactually high results (Slater, 1984). The appropriateness of the TBA assay for milk has been questioned (Ward, 1985). High-performance liquid chromatographic procedures are now available to determine malonaldehyde directly (Madere and Behrens, 1992). A gas chromatographic procedure is also available (Frankel and Neff, 1983).

King (1962) showed that the TBA method correlates well with the intensity of oxidized flavor in liquid milk. Downey (1969) suggested that the TBA procedure of Dunkley and Jennings (1951) is more applicable than that of King (1962) for determining the extent of the off-flavor.

Other traditional methods available for monitoring the extent of lipid oxidation include the Anisidine value, the Kreis test (Mehlenbacher, 1960), methods based on the carbonyl content of oxidized fats (Henick *et al.*, 1954; Lillard and Day, 1961), and measurement of oxygen uptake either by manometry or polarography (Tappel, 1955; Hamilton and Tappel, 1963).

In recent years, modern instrumental methods have been developed to monitor lipid oxidation in biological samples, including dairy products. These include use of electron spin resonance (ESR) spectrometry, direct measurement of secondary oxidative products such as malonaldehyde, static and dynamic GC/MS methods. ESR spectrometry permits detection of free radicals formed in the very early stages of oxidation prior to the formation of peroxides. The method has been applied successfully to dairy products such as milk powders and processed cheese (Nielsen *et al.*, 1997; Stapelfeldt

*et al.*, 1997a,b; Kristensen and Skibsted, 1999). Data indicate that the method correlates well with the TBA method and with sensory evaluation of oxidation in these dairy products. The method may have potential for application in quality control and accelerated testing of dairy products for oxidative stability.

The traditional TBA and anisidine value tests can be replaced by a qualitative or quantitative analysis of oxidation-derived volatiles using headspace GC/MS methodology or the so-called “electronic nose” apparatus. These techniques have been applied successfully to a variety of dairy products (Lee *et al.*, 1991; Park and Goins, 1992; Ulberth and Roubicek, 1995; Christensen and Holmer, 1996; Kim and Morr, 1996). For example, Kim and Morr (1996) reported that exposure of milk to fluorescent light for 48 h and subjected to dynamic headspace analysis resulted in the identification of five major volatile compounds: 2-butanone, 2-propanol, pentanal, dimethyl disulfide and hexanal. The classes of volatiles recovered were remarkably similar to those reported by Dimick (1982). Results of dynamic headspace analysis correlated well with sensory panel results. Frankel (1993) has stated that headspace analysis of volatile oxidation products by gas chromatography gives better information about the origin of flavor and odor volatiles than traditional chemical analyses and is the best method for comparison with sensory panel results.

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# Nutritional Significance of Milk Lipids

P.W. Parodi

## 17.1. Introduction

Fat is a concentrated source of energy that provides 30–40% of dietary calories in developed countries. Fat imparts palatability to food, serves as a vehicle for fat-soluble vitamins A, D, E and K and supplies essential fatty acids. The digestion products of fats, along with endogenously synthesized lipids, provide a diverse group of molecules that play a critical role in multiple metabolic processes.

Triacylglycerols are the major storage form of energy in animals. They also protect the body against both cold and heat loss and protect many organs from trauma due to external forces associated with day-to-day living. Lipids are vital components of cell membranes and take part in many inter- and intra-cellular signalling cascades. Lipids have multiple forms and functions, including vitamins, steroid hormones, and eicosanoids that are involved in many metabolic processes.

In the previous edition of this series, Gurr (1994) provided a chapter on the nutritional significance of lipids, which included aspects of their physiology and biochemistry. Since then, considerable advances have been made in lipid research, which Gurr *et al.* (2002) have covered recently in a very readable textbook. This chapter concentrates on the nutritional significance of milk lipids, a subject that has received scant attention in recent times. This omission is, no doubt, influenced by the negative nutritional image milk fat has acquired in recent decades due to its perceived association with coronary artery disease, and more recently to the belief that fat in general may be linked to cancer at some sites and to the current obesity epidemic.

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

## 17.2. Dietary Fat and Obesity

During the past two to three decades, the prevalence of obesity in most countries has increased dramatically. In affluent countries, it is estimated that 30% of adults are obese and another 35% are overweight. Apart from aesthetic concerns, excess adiposity can induce multiple metabolic abnormalities that contribute to a number of life-threatening diseases. Excess weight is responsible for 30–40% of heart disease, which no doubt occurs through linkage to other risk factors, such as hypertension, dyslipidemia, and type 2 diabetes with its associated insulin resistance and hyperinsulinemia. Other conditions associated with excess weight are cancer at several sites, including breast and colon, gall bladder disease, sleep apnea and an increased incidence of osteoarthritis (Willett, 2002).

Regional distribution of body fat can influence health risks. Subjects with upper-body obesity, where fat has accumulated in subcutaneous and visceral deposits are more prone to metabolic defects than those with peripheral obesity, where fat is stored in the buttock, thighs and lower abdomen (Lafontan and Berlan, 2003). Certain individuals have a genetic propensity to store excess energy as fat; others may harbour mutations in genes that regulate energy balance (Friedman, 2003). However, genetic changes are not responsible for the soaring incidence of obesity during the past 30 years. Rather, the incidence generally reflects an imbalance between energy intake and energy expenditure due to the combination of an abundant supply of cheap, palatable, energy-dense foods and an increasingly sedentary lifestyle.

The amount of fat in the diet has been blamed for the increase in obesity. There are two reasons for this. Firstly, the energy value of fat is 9.0 kcal/g compared to 4.0 kcal/g for protein and carbohydrate. Secondly, the prevalence of overweight individuals tends to be higher in affluent countries with a high fat intake than in underdeveloped countries with low fat intakes. However, these between-population (ecologic) comparisons are seriously confounded by differences in a number of lifestyle factors including types and availability of food and in physical activity. Moreover, the extensive increase in the incidence of obesity and overweight occurred during the period when there was a substantial decline in the percentage of energy derived from fat (Willett, 2002).

Within-population epidemiological studies can avoid confounding due to differences in lifestyle, ethnicity and food patterns found in ecological studies. Seidell (1998) and Willett (2002) have reviewed within-population studies on the relation between fat intake and obesity. Case-control studies have produced inconsistent results. This format can be prone to serious biases because obese subjects have been shown to underestimate their energy and fat intake by up to 50%. Non-obese subjects, on the other hand, tend to

have healthy lifestyles, aspects of which are difficult to control statistically. Prospective (cohort) studies should, but do not always, overcome these biases. Nevertheless, the outcomes of studies that investigated the association between percentage of energy from fat and weight gain were inconsistent. Thus, there is no conclusive evidence from epidemiological studies that intake of dietary fat promotes the development of obesity independently of total energy intake (Seidell, 1998; Willett, 2002).

### 17.2.1. Randomized Control Trials

The randomized clinical trial is the most reliable method for assessing the efficacy of diet or drug therapy. A number of systematic reviews have been conducted on randomized clinical trials that investigated low-fat diets versus other weight-reducing diets in overweight or obese individuals (Willett, 2002; Kris-Etherton *et al.*, 2002; Pirozzo *et al.*, 2003; Sanders, 2003).

Short-term dietary intervention studies on overweight and obese individuals show that diets with a lower percentage of calories from fat lead to modest weight losses (1–4 kg). However, in trials that lasted for one year or more, there were no significant differences in sustained weight loss between diets containing 18–40% of energy derived from fat and other weight-reducing diets. Furthermore, overall weight loss at the conclusion of these studies was of the order of only 2–4 kg. There is no evidence to suggest that low-fat diets are more beneficial than other diets of equal energy density for the prevention of weight regain.

A study using whole-body calorimetry showed that there was no significant difference in net fat accumulation in lean or obese women when fed controlled excess of dietary energy supplied by the monosaccharides glucose and fructose, the disaccharide sucrose, or fat (McDevitt *et al.*, 2000).

### 17.2.2. Safety of Low-Fat, High-Carbohydrate Diets

Low-fat, high-carbohydrate diets have been shown to lower plasma low-density lipoprotein (LDL) cholesterol levels. At the same time, these diets decrease anti-atherogenic high-density lipoprotein (HDL)-cholesterol, and increase concentrations of plasma triglycerides, lipoprotein[a] (Lp[a]), and small dense LDL, plus increasing insulin resistance. Overall, these changes are likely to increase the risk of coronary heart disease (Willett, 2002; Kris-Etherton *et al.*, 2002; Sanders, 2003).

### 17.2.3. Energy Value of Milk Fat

The energy value of a fatty acid is dependent on the ratio of its carbon atom content to its oxygen atom content. Stearic acid (C<sub>18:0</sub>) has a high

carbon to oxygen ratio and the heat of combustion, used to calculate the energy value, is 9.48 kcal/g. On the other hand, butyric acid (C<sub>4:0</sub>) has a low carbon to oxygen ratio and a heat of combustion of 5.92 kcal/g.

Furthermore, after digestion of milk fat, the fatty acids with chain lengths of less than 12 carbon atoms are absorbed from the intestine, pass to the portal circulation and are transferred to the liver, where they are rapidly metabolized by  $\beta$ -oxidation. In contrast, the longer-chain fatty acids are resynthesized into triacylglycerols in the enterocytes and are packed into chylomicrons that enter the lymph and are transported up the thoracic duct and emptied into the venous circulation. In the capillaries of extra-hepatic tissues, but mainly adipose tissue and muscle, lipoprotein lipase hydrolyses triacylglycerols of chylomicrons to fatty acids. Muscle cells consume these acids as fuel, whereas adipocytes incorporate them into triacylglycerols for energy storage. When triacylglycerols are required for energy their fatty acids are degraded by  $\beta$ -oxidation to acetyl-CoA and then to ATP. For saturated fatty acids, oxidation decreases with increasing carbon chain-length.

Up to one-third of the fatty acids in milk fat have a chain-length of 14 carbons or less. Because these acids are oxidized rapidly in the liver, have a lower energy value and are oxidized more readily than long-chain fatty acids, it follows that milk fat should contribute less to overweight than an equivalent amount of other dietary fats (Parodi, 2004). A study by Schneeman *et al.* (2003) showed that milk fat is a more potent stimulator of cholecystokinin than a blend of non-milk fat with a similar ratio of polyunsaturated to saturated fatty acids. Cholecystokinin is a “satiety” hormone released into the blood stream by the intestine during feeding and acts to suppress further eating.

### 17.3. Dietary Fat and Cancer

As recently as the early 1980s, public health organizations were recommending a reduction in fat intake for the prevention of cancer. This recommendation was based largely on evidence from early animal studies and from international comparison studies, which showed strong correlations between national *per capitum* fat consumption and the incidence of cancer (Willett, 2001a; Kushi and Giovannucci, 2002). However, other aspects of Western lifestyles, including positive energy balance and physical inactivity, seriously confound ecological studies; moreover, population correlations tell nothing about the diet of individuals who get cancer and those who do not.

On the other hand, within-population studies of a cross-sectional format have yielded conflicting results, but large prospective studies, which

provide the most rigorous evidence of association, have not supported an important role for total fat intake and cancer (Willett, 2001a; Kushi and Giovannucci, 2002). A brief outline of the role of dietary fat in the development of colon (colorectal), breast and prostate cancers—the major non-smoking related malignancies—follows. It should be appreciated that fat or a sub-type of fat is usually not consumed as a single unit, but rather as a component of a food item, that may contain both pro- and anti-cancer agents. For example, dairy products contain calcium, whey proteins as well as lipid components with anti-cancer potential (Parodi, 2001b).

### 17.3.1. Colon Cancer

International comparative studies show a strong correlation between *per capitum* disappearance of fat and rates of colon cancer (Willett, 2001a; Kushi and Giovannucci, 2002). Early animal studies suggested that dietary fat plays an important role in the initiation and promotion of colon tumorigenesis. However, later evidence showed that total energy intake, rather than fat intake, was more likely to influence tumor development (Howe *et al.*, 1997).

A number of case-control studies have shown an association between the risk of colon cancer and intake of fat. In many of these studies, a positive association between total energy intake and the risk of colon cancer was also noted, which is important because of the high correlation between fat intake and energy consumption. Other studies showed that physical inactivity or excess energy intake relative to requirements strongly increased the risk of this malignancy (Giovannucci and Goldin, 1997). Howe *et al.* (1997) combined the data from 13 case-control studies and found a significant association between total energy and the risk of colorectal cancer. However, neither total, saturated, mono- nor poly-unsaturated fat was associated with risk of colorectal cancer independent of total intake of energy.

Three of the four large prospective studies that adjusted for energy intake, the Netherlands Cohort Study (58,279 men, 62,573 women), the Iowa Women's Health Study (35,215 women), and the Health Professionals Follow-up Study (47,949 men), did not find an association between total fat intake or class of fat and the risk of colon cancer. The fourth study, the Nurses' Health Study (88,751 women) showed that animal fat, but not vegetable or fat from dairy products, was positively associated with the risk of colon cancer. Because intake of red meat has been associated with colon cancer in a number of studies, the increased risk may be linked to compounds like haem and heterocyclic amines formed during high-temperature cooking of meat rather than to fat (Giovannucci and Goldin, 1997; Willett, 2001a; Kushi and Giovannucci, 2002).

Overall, epidemiological evidence from within-population studies does not support an independent role for dietary fat or fat subclasses in the risk of colon cancer. However, the evidence does not preclude the possibility that certain fatty acids, such as  $\omega$ -6s and  $\omega$ -3s, may exert opposing influences.

### 17.3.2. Breast Cancer

There is an extensive literature on the association between fat intake and the risk of breast cancer. Many animal studies showed that diets high in fat are associated with an increased incidence of chemically induced tumor development. However, these associations may be due to the effect of total energy intake. The study of Ip *et al.* (1990), designed specifically to determine the effect of fat, independent of energy intake, showed that:

- Mammary tumorigenesis was very sensitive to the level of linoleic acid in the diet and increased proportionally in the range of 0.5–4.4% at which the effect leveled off
- There may be a small effect of fat distinct from the calorie effect
- Calorie restriction is more striking than a decrease in dietary fat in suppressing tumor development
- Reducing calorie intake can interrupt the promotion of mammary cancer by a high fat diet

High intake of total fat is correlated with an increased incidence of breast cancer in international comparative studies. In addition to the usual factors that confound associations between dietary fat and cancer in this type of study, countries with a high fat intake also have a lower age at menarche, later age at first birth, lower parity and higher post-menopausal body weight, which are risk factors for breast cancer (Willett, 2001b; Kushi and Giovannucci, 2002).

Evidence from case-control studies for an association between total fat intake and the risk of breast cancer is inconsistent (Willett, 2001b; Kushi and Giovannucci, 2002). On the other hand, prospective studies offer no support for an association between breast cancer risk and intake of total fat or specific types of fat. Hunter *et al.* (1996) conducted a collaborative-pooled analysis of original data from seven large prospective studies published up to 1995 that represented 4980 cases. The analysis found no evidence of an association between the intake of total, saturated, mono- or poly-unsaturated fat and the risk of breast cancer. There was no reduction in risk even among women whose energy intake from fat was less than 20% of total energy intake. What is more, for the small number of women reporting less than 15% energy from fat, the risk of breast cancer increased more than

twofold. A follow-up pooled analysis by Smith-Warner *et al.* (2001), which included 7,329 cases, supported the lack of association between total fat, fat class, animal or vegetable fat intake and the risk of breast cancer.

The 14-year follow-up of the Nurses' Health Study, the largest of the prospective studies with 2,956 cases, also found no evidence of association between total or type of fat and breast cancer risk (Holmes *et al.*, 1999a). Further, no survival advantage was found for a low-fat diet or diets containing a particular fat type after diagnosis of breast cancer in this cohort of nurses (Holmes *et al.*, 1999b).

### 17.3.2.1. Specific Fatty Acids

Opposing effects of certain individual fatty acids could have influenced the lack of a relationship between dietary fat and fat type with the risk of breast cancer. Well-conducted animal studies suggest that linoleic acid promotes development of mammary tumors, whereas saturated, monounsaturated, and trans fatty acids have little or no effect. In many cases,  $\omega$ -3 polyunsaturated fatty acids suppress tumor development. Conjugated linoleic acid (CLA) is the most potent anti-cancer fatty acid in that amounts of 1% or less of dietary fat can substantially inhibit the development of mammary tumors (Ip, 1997).

Nevertheless, at this time there is no persuasive evidence from epidemiological studies that any individual fatty acid is associated with the risk of breast cancer (Willett, 1997). A pooled analysis of nine prospective studies showed no association between the intake of various dairy products and the risk of breast cancer (Missmer *et al.*, 2002). However, in epidemiological studies there is often a high degree of correlation between individual fatty acids in the diet. This reduces the ability to detect an independent association between a single acid and cancer risk. Furthermore, dietary assessment during an epidemiological study may not reflect an individual's diet at the time of cancer initiation, which in the case of breast cancer may be in early life.

### 17.3.3. Prostate Cancer

Similar to cancer at other sites, there are strong correlations between *per capitum* fat intake and prostate cancer deaths in international comparative studies (Kolonel, 2001; Kushi and Giovannucci, 2002). Early case-control studies often showed positive associations between prostate cancer risk and total, animal, and saturated fat intake. However, in later studies in which energy intake was adjusted, only two of five found an increased risk for total fat intake, whereas two of eight found an increase in the risk of prostate cancer with animal fat intake (Kolonel, 2001).

There have been a number of prospective studies in which the relationship between fat intake and risk of prostate cancer was examined. Only four of these studies adjusted for energy intake. In these studies, there were no statistically significant associations between total fat, fat type or individual fatty acids and the risk of prostate cancer. An exception was a positive association with  $\alpha$ -linolenic acid in the Health Professionals Follow-up Study (Kolonel, 2001).

Unlike colon and breast cancer, there is a lack of suitable animal models for mechanistic and dietary studies on prostate cancer. Studies that have investigated dietary fat intake and the role of individual fatty acids on prostate cancer risk have produced inconsistent results.

#### 17.3.4. Comment

Evidence from well-conducted epidemiological studies does not support any meaningful associations between the intake of total fat, fat type or individual fatty acids and the risk of colon, breast or prostate cancer. Final proof of a null effect should come from randomized clinical trials. However, such trials seem unlikely because initiation of tumors may occur early in life, whereas the clinical symptoms arise late in life. The cost of appropriate trials would be prohibitive. Further advances, however, may come from improved dietary assessment and a better understanding of, and adjustment for, confounding factors in epidemiological studies.

### 17.4. Milk Fat and Coronary Heart Disease

By far the most telling negative nutritional aspect of milk fat is the belief that its content of saturated fatty acids and cholesterol elevate plasma cholesterol levels, which is a risk factor for coronary heart disease (CHD). During the early 1950s, it was found that the type of fat in the diet could influence plasma cholesterol level. Ahrens *et al.* (1957) showed that diets containing saturated fats, such as beef, lard and milk fat, produced higher plasma cholesterol levels than diets containing unsaturated fat like safflower and corn oil when they were fed under strict metabolic-ward conditions. Later, Connor (1961) reported that the level of cholesterol in the diet also influenced plasma cholesterol level.

Since then, there have been numerous studies that investigated the effect of different types and amounts of fat, individual fatty acids and other dietary components on plasma cholesterol level. It is now realized that all saturated fatty acids do not elevate plasma cholesterol levels to the same extent. The short-chain fatty acids, butyric (C<sub>4:0</sub>), caproic (C<sub>6:0</sub>), caprylic (C<sub>8:0</sub>); the medium-chain capric (C<sub>10:0</sub>); and stearic (C<sub>18:0</sub>) acids,

like monounsaturated acids, have no discernible effect on plasma cholesterol level. On the other hand, the relative hypercholesterolemic effect of lauric (C<sub>12:0</sub>), myristic (C<sub>14:0</sub>) and palmitic (C<sub>16:0</sub>) acids is controversial, but myristic acid may exert the greatest potency (Mensink *et al.*, 2003).

#### 17.4.1. Plasma Cholesterol and CHD

A number of studies, including several large prospective studies, such as the Framingham Study (Anderson *et al.*, 1987), the Multiple Risk Intervention Trial (Stamler *et al.*, 1986) and the Lipid Research Clinics Program (Pekkanen *et al.*, 1990), as well as the Seven Countries Study (Verschuren *et al.*, 1995) showed a positive correlation between levels of plasma cholesterol and mortality from CHD. However, epidemiological associations cannot prove causality and elevated cholesterol levels could be either a cause, a correlate or a consequence of CHD.

The early studies on CHD demonstrated that saturated fatty acids and cholesterol increase, whereas polyunsaturated fatty acids decrease plasma cholesterol levels, and that cholesterol levels are associated with CHD risk, led to the diet-heart or lipid hypothesis of CHD. Conversely, it followed that lowering the intake of saturated fatty acids and increasing polyunsaturated fatty acid intake will lower plasma cholesterol levels, which in turn will reduce the risk of CHD.

The studies that led to the lipid hypothesis measured plasma total cholesterol concentration. Cholesterol is insoluble in aqueous solution and needs to be combined with protein for transport in blood. These plasma lipoproteins are large heterogeneous aggregates that have different physical properties, such as density, chemical composition and metabolic function (Gurr *et al.*, 2002).

Later epidemiological studies demonstrated that the low-density lipoprotein (LDL)-cholesterol, which is the predominant cholesterol carrier, like total cholesterol, was positively associated with the risk of CHD. On the other hand, HDL-cholesterol was negatively associated with the risk of CHD. Even between individuals having the same LDL-cholesterol level, those with a predominance of small, dense LDL particles have a much higher risk of CHD than individuals with a predominance of large, buoyant LDL particles (Gurr *et al.*, 2002). It is notable that the C<sub>12:0</sub>, C<sub>14:0</sub> and C<sub>16:0</sub> fatty acids that increase total and LDL-cholesterol the most, concomitantly increase the levels of anti-atherogenic HDL-cholesterol, such that there can be a beneficial decrease in the total:HDL ratio (Mensink *et al.*, 2003).

Unfortunately, early studies that measured only levels of total cholesterol are still cited in reviews (e.g., Braunwald, 1997; Schaefer, 2002) to support the contention that restricting saturated fat and cholesterol intake

and increasing the intake of polyunsaturated fatty acids will reduce the risk of CHD. In addition, Ravnskov (1992) has pointed out that reviews supporting the lipid hypothesis written by distinguished scientific bodies often exhibited bias by ignoring non-supportive studies.

Since the time the lipid hypothesis was proposed, a number of other risk factors, that can be modified, for CHD have been reported. Of importance are hypertension, cigarette smoking, obesity and physical inactivity, diabetes mellitus, and elevated plasma levels of Lp(a) and homocysteine (Braunwald, 1997; Schaefer, 2002).

Data from the 25-year follow-up of the much-cited Seven Countries Study (Verschuren *et al.*, 1995) showed that the level of plasma total cholesterol was linearly related to CHD mortality in all participating countries. However, the absolute levels of CHD mortality were strikingly different. At a serum cholesterol level of 5.2 mmol/L there was a fivefold greater mortality in Northern Europe than in Japan. These differences in mortality risk were established after adjusting for age, smoking and systolic blood pressure. This suggests that there are other powerful, as yet largely unknown, risk factors for CHD in industrialized communities. The WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) Project measured CHD events and risk factors for 38 populations in 21 countries during a 10-year period (Kuulasmaa *et al.*, 2000). The association between classic risk factors—namely, cigarette smoking, systolic blood pressure, BMI, and serum cholesterol—and trends in the incidence of coronary events was extremely weak or nonexistent.

Braunwald (1997) points out that fully half of all patients with CHD do not have any of the conventional risk factors (hypertension, hypercholesterolemia, cigarette smoking, diabetes mellitus, marked obesity and physical inactivity). Further, up to two-thirds of patients with CHD have what may be considered normal serum cholesterol levels (see references in Parodi, 2004). These facts suggest that the role of plasma cholesterol in CHD has been overemphasized and oversimplified.

### **17.4.2. Saturated Fatty Acids and CHD**

Notwithstanding the fact that the consumption of certain saturated fatty acids can increase plasma cholesterol level, which is a risk factor for CHD, is there any direct evidence to indicate that saturated fatty acids are associated with the risk of CHD?

#### **17.4.2.1. Epidemiology—International Comparative Studies**

Most ecological studies showed a strong positive correlation between *per capitum* disappearance rates of dietary fat, dairy fat, saturated fat, or

cholesterol and mortality from CHD when simple regression analysis was employed (Ravnskov, 1998; McNamara, 2000; Schaefer, 2002). The inappropriateness of this type of study for attributing causality has been discussed in earlier sections.

#### 17.4.2.2. Epidemiology–Case-Control Studies

Only a few case-control studies have investigated the association between CHD and the intake of saturated fat, polyunsaturated fat or total fat by CHD patients and by subjects free from CHD. Ravnskov (1998) lists six studies, none of which supported the hypothesis that saturated fat is associated with an increased risk, and polyunsaturated fat with a decreased risk of CHD.

#### 17.4.2.3. Epidemiology–Prospective Studies

Ravnskov (1998) presented data for 28 cohorts from 21 prospective studies. In only three of these cohorts did the evidence show that saturated fat was associated with a statistically significant increased risk of CHD. CHD patients in three cohort studies had consumed significantly more polyunsaturated fat, and in only one cohort had CHD patients eaten less polyunsaturated fat than CHD-free participants. The cohorts included the Framingham Study and the large well-conducted Health Professionals Follow-up Study and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Since then, Hu *et al.* (1997) presented the 14-year follow-up data from the Nurses' Health Study. After adjustment for confounding variables in multivariate analyses, no statistically significant associations were found between intake of total fat, animal fat, or saturated fat and the risk of CHD.

Later, Hu *et al.* (1999) re-analyzed the fatty acid data from the 14-year follow-up results of the Nurses' Health Study. The saturated fatty acids were grouped as  $C_{4:0} - C_{10:0}$ ,  $C_{12:0} + C_{14:0}$ ,  $C_{16:0}$ ,  $C_{18:0}$ , and  $C_{12:0} - C_{18:0}$ . After adjustments for confounding variables in multivariate analyses, none of the saturated fatty acid groupings was positively associated with the risk of CHD. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study also investigated the relationship between specific groups of saturated fatty acids and CHD risk (Pietinen *et al.*, 1997). After multivariate adjustment for energy and other confounding risk factors, the study found statistically significant inverse associations between the risk of CHD mortality and the intake of total saturated fatty acids and the so-called hypercholesterolemic fatty acids ( $C_{12:0} - C_{16:0}$ ). What is more, this study showed positive associations between intake of polyunsaturated fatty acids and linoleic acid and the risk of death from CHD.

Epidemiological studies provide little, if any, evidence to support the hypothesis that saturated fatty acids, even those of chain length  $C_{12:0} - C_{16:0}$  that can elevate serum cholesterol concentration, are associated with the risk of CHD. This may result from the increased plasma HDL-cholesterol concentration produced by saturated fatty acids largely compensating for the adverse effects of these fatty acids on LDL-cholesterol concentration (Hu and Willett, 2000). In addition, saturated fatty acids lower the level of plasma Lp[a], which is considered a significant risk factor for CHD (Mensink *et al.*, 1992).

### 17.4.3. Dietary Cholesterol and CHD

#### 17.4.3.1. Dietary Cholesterol and Serum Cholesterol

Clinical studies show that dietary cholesterol is a less potent regulator of plasma cholesterol than are saturated fatty acids. Results from meta-analyses predict that plasma cholesterol response to a 100 mg/day change in dietary cholesterol will be from 0.06 to 0.07 mmol/L. The data show that although dietary cholesterol elevates plasma total cholesterol and LDL-cholesterol level, it also increases the level of HDL-cholesterol such that there is little overall effect on the LDL:HDL ratio (McNamara, 2000).

#### 17.4.3.2. Dietary Cholesterol and the Epidemiology of CHD

Numerous international comparative studies found significant positive correlations between *per capitum* consumption of cholesterol and CHD mortality using simple univariate regression analysis (McNamara, 2000).

Ravnskov (1995) listed cholesterol intake for CHD patients and controls from 14 within-country longitudinal studies. In only two of these studies were CHD patients found to have a statistically significantly higher intake of cholesterol than control subjects.

Kritchevsky and Kritchevsky (2000) provided a summary of the evidence linking dietary cholesterol to the risk of CHD in 10 cohorts from eight large, well-conducted prospective studies that were reported since 1980, which included the Nurses' Health Study, the Health Professionals Follow-up Study and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. In eight of the cohorts there was no statistical association between cholesterol intake and the risk of CHD. In one of the positive studies the association was established by simple univariate analysis and was not adjusted for other dietary variables. The other study adjusted only for fat intake. There is no compelling evidence from these epidemiological studies that dietary cholesterol is associated with the risk of CHD.

#### 17.4.4. Intervention Studies for CHD Prevention

A consequence of the lipid hypothesis of CHD is that reduction of saturated fat and cholesterol and an increase of polyunsaturated fatty acids in the diet will lower plasma cholesterol level with a reduction in the risk of CHD. A causal relation between dietary fat and CHD can be established only by conducting randomized clinical trials.

##### 17.4.4.1. Dietary Intervention Trials

There have been three primary and eight secondary prevention trials in which dietary change was the only variable. Dietary modification included reduction in total fat, substitution of saturated fat by polyunsaturated oils and reduction in cholesterol intake. These changes resulted in a reduction of saturated fat intake by 27–55% and reductions in plasma cholesterol of up to 18%. However, with the exception of one study, the Lyon Diet Heart Study (de Lorgeril *et al.*, 1994), neither total or CHD mortality was lowered significantly by the dietary interventions (Ravnskov, 1998; Parodi, 2004). In the successful Lyon Diet Heart Study, a Mediterranean-type diet was compared with the usual post-infarct prudent diet. Throughout this trial, plasma cholesterol levels were similar in both the treatment and control groups.

##### 17.4.4.2. Multifactor Intervention Trials

Multiple studies have examined the effect of dietary modification plus various lifestyle changes or drug therapy on CHD outcome and total mortality. Muldoon *et al.* (1990), Ravnskov (1992), and Davey Smith *et al.* (1993) conducted meta-analyses of these trials. Overall, there was a small benefit for nonfatal CHD, which was more noticeable in high-risk patients, but not for CHD or total mortality.

##### 17.4.4.3. Drug Intervention Trials

Bucher *et al.* (1999) conducted a systematic review of the benefit of different classes of cholesterol-lowering drugs for prevention of CHD and total mortality. Only the statin drugs showed a statistically significant reduction in mortality from CHD and from all causes.

*Statin Drugs and CHD.* Statin drugs are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that act by inhibiting HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis. The statins are now the most commonly prescribed drugs for the treatment of hypercholesterolemia. They can reduce plasma total cholesterol by 20–42%, LDL-cholesterol by 25–55% and triglycerides by 10–35%. In addition,

HDL-cholesterol levels are increased by 4–8% (Maron *et al.*, 2000). These changes in plasma lipids are claimed to reduce the risk of major coronary events by around 30%. However, this value is misleading because it refers to the relative risk of CHD; the absolute risk reduction is only about 2%.

Parodi (2004) listed the six major statin intervention trials of five years duration or longer. The absolute risk reduction in CHD mortality ranged from 0.12 to 3.5% and in total mortality from –0.1 to 3.3% with weighted means of 1.3 and 1.7%, respectively. In the statin trials, the relative reduction in risk was independent of baseline levels of LDL-cholesterol (Sacks *et al.*, 2000), whereas the absolute or percentage reduction in LDL-cholesterol had little relationship to coronary events (Sacks *et al.*, 1998). The risk of CHD events with statin therapy is considered less than with other drugs that produce comparable reductions in plasma cholesterol level. Moreover, angiography studies suggest that clinical improvement with statin therapy far exceeds changes in the size of atherosclerotic lesions (Takemoto and Liao, 2001).

It is now realized that statin drugs possess a number of important anti-atherogenic properties, independent of reduction in cholesterol level. Statin therapy can improve endothelial function, decrease oxidative stress and vascular inflammation and improve the stability of atherosclerotic plaques (Maron *et al.*, 2000; Takemoto and Liao, 2001). Because of these pleiotropic effects, the contribution of reduction in plasma cholesterol level, if any, to reduction in the risk of CHD cannot be assessed and cannot be used to validate the lipid hypothesis. It is relevant that the hypolipidemic drug gemfibrozil and agents like calcium channel blockers,  $\beta$ -adrenergic blockers, angiotensin-converting enzyme inhibitors, and aspirin can reduce the risk of CHD events to about the same extent as statins without lowering plasma cholesterol levels (Parodi, 2004).

#### 17.4.5. Comment

Evidence from well-conducted prospective epidemiological studies does not suggest that consumption of saturated fat and cholesterol is associated with an increased risk of CHD. Randomized clinical trials that reduced the intake of saturated fatty acids and cholesterol and increased the intake of polyunsaturated fatty acids to lower plasma cholesterol levels did not significantly improve CHD or total mortality. The minor improvement in CHD events for trials of the potent cholesterol-lowering statin drugs may result, to an unknown extent, from their pleiotropic effects and cannot be used to justify the lipid hypothesis.

Unfortunately, when saturated fatty acids are replaced by carbohydrate in the so-called low-fat, high-carbohydrate diets, a reduction in plasma

LDL-cholesterol levels is accompanied by an increase in small, dense LDL particles, triglycerides and insulin and a decrease in HDL-cholesterol level (Krauss, 2001), all of which are risk factors for CHD.

Individuals do not consume saturated fatty acids and cholesterol as dietary items, rather they are components of milk fat and other lipids and milk fat contains other components such as, sphingolipids and ruminic acid (RA; *cis*-9, *trans*-11-C<sub>18:2</sub>), which may help prevent CHD. Similarly, milk fat is consumed as a component of dairy products and dairy product consumption has been associated with reduction in blood pressure, weight loss and a reduced incidence of the metabolic syndrome (a convergence of insulin resistance, glucose intolerance, hypertension, obesity and dyslipidemias), which are CHD risk factors (Parodi, 2004). Indeed, the Nurses' Health Study found no significant associations between intake of low-fat or high-fat dairy products and major CHD events (Hu *et al.*, 1999), whereas a recent prospective case-control study found that the estimated intake of milk fat was negatively associated with the risk of CHD (Warensjo *et al.*, 2004). Elwood *et al.* (2004) identified ten prospective epidemiological studies that measured milk consumption and cardiovascular disease. A pooled estimate of risk found that drinking milk was associated with a small but worthwhile reduction in CHD and stroke risk.

## 17.5. *Trans* Fatty Acids and CHD

Early investigations into a link between dietary *trans* fatty acid (TFA) intake and plasma cholesterol level in clinical trials, and CHD in epidemiology studies provided conflicting results. This outcome resulted from small numbers of participants in the trials combined with poor experimental design.

### 17.5.1. Clinical Studies

In the early 1990s, a series of well-designed clinical studies convincingly demonstrated that TFAs increased plasma total and LDL-cholesterol to levels similar to those produced by saturated fatty acids. More than this, TFAs reduced plasma HDL-cholesterol level. The overall effect was that the ratio of LDL-cholesterol to HDL-cholesterol was approximately double that for an equivalent intake of saturated fatty acids (Ascherio *et al.*, 1999). In addition, TFAs adversely affect other CHD risk factors. Plasma triglycerides and Lp[a] levels are increased (Ascherio *et al.*, 1999) and it was shown recently that consumption of TFAs was associated with a deleterious increase in small, dense LDL particles (Mauger *et al.*, 2003).

## 17.5.2. Epidemiological Studies

Early studies of the relation between intake of TFAs and the occurrence of CHD also produced conflicting results. However, the large, well-conducted prospective studies showed positive associations between TFA intake and risk of CHD. The Nurses' Health Study and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study produced statistically significant positive associations, whereas in the Health Professionals Follow-up Study the positive association did not attain statistical significance (Ascherio *et al.*, 1999). There was also a significant positive association between TFA intake and CHD risk in the smaller Zutphen Elderly Study (Oomen *et al.*, 2001).

### 17.5.2.1. Differential Effect of Ruminant TFAs and TFAs from Hydrogenated Vegetable Oils

A case-control study (Ascherio *et al.*, 1994), a cross-sectional study (Bolton-Smith *et al.*, 1996) and three prospective studies; the Nurses' Health Study (Willett *et al.*, 1993), the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (Pietinen *et al.*, 1997), and the Zutphen Elderly Study (Oomen *et al.*, 2001), separately assessed the effect of TFAs from hydrogenated vegetable oil and animal fat on the risk of CHD. With the exception of the small Zutphen Elderly Study (Oomen *et al.*, 2001), the studies found that the positive association with the risk of CHD was explained entirely by the intake of TFAs from hydrogenated vegetable oil.

## 17.5.3. Biological Explanation for the Disparate Effects

### 17.5.3.1. Dietary TFAs

About one-fourth of the TFAs in ruminant fat is represented by RA, which is by far the predominant natural isomer of conjugated linoleic acid (CLA). The remaining TFAs are mainly *trans* monounsaturated acids of which vaccenic acid (VA; *trans*-11-C<sub>18:1</sub>) is predominant. On the other hand, hydrogenated vegetable oils contain predominantly elaidic acid (*trans*-9-C<sub>18:1</sub>) and have a more even distribution of the other *trans*-C<sub>18:1</sub> acids, but contain little or no RA (Parodi, 2004).

### 17.5.3.2. Bioconversion of VA to RA

Animal and human tissues contain the enzyme  $\Delta^9$ -desaturase that can introduce a *cis*-double bond at carbon 9 in VA to produce RA. Santora *et al.* (2000) fed VA to mice and found that 12% of this VA or 50% of the VA stored in carcass adipose tissue was converted to RA. Humans fed increasing

amounts of VA exhibited linear increases of RA in their plasma. The mean conversion level was about 20% (Turpeinen *et al.*, 2002). Because the content of VA in ruminant fat can be several-fold higher than the content of RA, bioconversion of VA may effectively double the supply of RA to target tissues.

#### 17.5.4. RA and Atherosclerosis

Several studies on animal models demonstrated that CLA could retard the development of atherosclerosis. The CLA used in these studies was a mixture of isomers in which RA and *trans*-10, *cis*-12-C<sub>18:2</sub> were predominant and present in near equal amounts. Because these two isomers can show identical biological effects in some tissues and dissimilar effects in other tissues, a benefit for RA in these studies cannot be assumed. Nevertheless, there is emerging evidence that RA has anti-atherogenic properties.

Kritchevsky (1999) reviewed studies conducted with colleagues, which demonstrated that dietary CLA could inhibit the development of cholesterol-induced atherosclerosis in hamsters and in rabbits. Reduction in the severity of pre-existing lesions was also noted. Recently, Kritchevsky (2003) fed rabbits a diet containing 1% of relatively pure RA. This diet significantly inhibited the formation of atherosclerotic lesions in the aortic arch and thoracic aorta, compared to a diet without RA.

The validity of extrapolating observations on animal models to human atherosclerosis is often questioned. However, during the last decade gene deletion technology has allowed the production of a variety of transgenic or knockout animal models that can closely resemble particular human lipoprotein disorders. One model that is now used extensively to study atherosclerosis is the apolipoprotein E-deficient mouse (apo E<sup>-/-</sup>). In this model, mice develop severe hypercholesterolemia and atherosclerosis on a regular low-fat, low-cholesterol diet. The progression and histopathology of the lesions are similar to those that develop in humans. Recently, Toomey *et al.* (2003) reported that 1% RA fed to apo E<sup>-/-</sup> mice not only inhibited the development of atherosclerosis but also caused the regression of established lesions.

In most cases, the reduced incidence of atherosclerosis in animals fed CLA or RA was not accompanied by a decrease in plasma total or LDL-cholesterol level or an increase in HDL-cholesterol level. Thus, other mechanisms must be responsible for the prevention of atherosclerosis. Atherosclerosis is recognized nowadays as a chronic inflammatory disease, and RA has been shown to exhibit a number of important anti-inflammatory properties (Yu *et al.*, 2002):

- It reduces the expression of the inducible form of cyclooxygenase (COX)-2 and the end product prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). COX-2 is a rate-limiting enzyme in the conversion of arachidonic acid to series-2 prostacyclins, the vasoconstricting thromboxanes, and prostaglandins such as PGE<sub>2</sub>.
- It decreases the production of inducible nitric oxide synthase, which is responsible for the release of pro-inflammatory nitric oxide.
- It reduces the production of the pro-inflammatory cytokines tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ), interleukin (IL)-1, and IL-6.

RA was also shown to inhibit the production of series-2 prostaglandins and thromboxanes in endothelial cells from human saphenous vein, which is the vein commonly used in coronary bypass surgery (Urquhart *et al.*, 2002).

These anti-inflammatory properties, as well as certain other biological properties of RA, are similar to those seen with ligands for the peroxisome proliferator-activated receptors (PPARs), especially the PPAR $\gamma$  isoform. The PPARs are nuclear receptors primarily involved in the regulation of lipid and glucose homeostasis. Activation of PPAR $\gamma$  inhibits intracellular signalling cascades, such as for nuclear factor  $\kappa$ B (NF- $\kappa$ B), a transcription factor that regulates cytokine production. A number of synthetic drugs used in the treatment of dyslipidemias, diabetes and obesity, that are risk factors for CHD, are PPAR agonists. At the molecular level PPAR agonists can regulate foam cell formation, plaque stability, and decrease the formation of pro-atherosclerotic proteins, such as fibrinogen and C-reactive protein (Desvergne and Wahli, 1999; Kersten *et al.*, 2000).

RA is a potent activator of PPAR $\alpha$  and PPAR $\gamma$  (Belury *et al.*, 2002). Yu *et al.* (2002) found that inhibition of pro-inflammatory products by RA was associated with PPAR $\gamma$  activation. Likewise, Toomey *et al.* (2003) showed that the regression of preestablished atherosclerosis in apo E<sup>-/-</sup> mice fed RA is associated with an increased expression of PPAR $\gamma$ .

## 17.6. Anti-Cancer Agents in Milk Fat

The demonization of fat, especially milk fat, over the past several decades means that the diet has been deprived of several components with anti-cancer potential. Milk fat components, such as RA, sphingolipids, butyric acid, certain branched chain fatty acids, ether lipids, vitamins, and novel components introduced from feed, have been shown to prevent tumor development at a number of sites in animal models.

### 17.6.1. Rumenic Acid

#### 17.6.1.1. Early Studies with Mixed Isomers of CLA

During the past 15 years, multiple studies have shown that CLA could inhibit the development of carcinogenesis in various experimental models. Physiological concentrations of CLA suppressed the proliferation in a wide range of human cancer cell lines that included breast, ovarian, prostate, colon, liver, mesothelioma, glioblastoma and leukemia. This growth suppression by CLA was in contrast to linoleic acid, which usually promoted growth. Mice fed CLA and inoculated with human breast or prostate cancer cells had reduced tumor growth and drastic reduction in metastases. CLA also inhibited chemically-induced skin, stomach and intestinal tumors (Parodi, 2004). However, the outstanding attribute of CLA is its ability to inhibit mammary tumor development (Ip *et al.*, 2003).

In the initial study by Ip *et al.* (1991), the authors fed rats a standard diet or that diet supplemented with 0.5, 1.0 or 1.5% CLA, 2 weeks prior to and following administration of the carcinogen 7, 12-dimethylbenz[*a*]anthracene (DMBA). At the end of the experiment, the total number of mammary adenocarcinomas in the groups fed 0.5, 1.0 and 1.5% CLA was reduced by 32, 56 and 60% compared to the control, respectively. Tumor incidence, tumor multiplicity (number of tumors per rat) and total tumor weight were reduced to a similar degree. This tumor inhibition by CLA is in contrast to linoleic acid, which promotes tumor development in this model.

This group subsequently conducted numerous studies to explore mechanisms contributing to the anti-cancer action of CLA (reviewed in Ip *et al.*, 2003). Notable findings were:

- At low carcinogen doses, there was effective inhibition of tumor development when the diet was supplemented with as little as 0.05% CLA.
- CLA was effective for both the direct-acting carcinogen methyl-nitrosourea (MNU) and the indirect-acting DMBA, suggesting the action of CLA was independent of carcinogen activation.
- CLA was equally effective in inhibiting mammary tumor development when it was part of a 5% low-fat diet or a 20% high-fat diet.
- Tumor inhibition was similar when CLA was part of 20% unsaturated-fat diet provided as corn oil or as a 20% saturated-fat diet provided by lard.
- Even though a diet containing 12% linoleic acid produced more mammary tumors than a diet containing 2% linoleic acid, CLA suppressed tumor development to the same degree with both diets.

- CLA was preferentially incorporated into the neutral lipids of mammary tissue. When CLA was removed from the diet, its disappearance from neutral lipids paralleled the rate of occurrence of new tumors.

The age of the rat when CLA supplementation is commenced can influence outcome. When rats were fed CLA from weaning at 21 days of age until day 51 only, then administered a carcinogen at day 57, they were protected from subsequent tumor development. However, when CLA entered the diet for the same period of time after carcinogen administration and when the animals were older, there was no protection against tumor development. A continuous intake of CLA was then necessary to obtain equivalent protection. The period from 21 to 51 days of age corresponds to development of the mammary gland to adult stage morphology.

Further studies showed that during the pubescent period, CLA reduced the development and branching of the expanding mammary ductal tree. There was also a reduction in the density and rate of proliferation of terminal end bud (TEB) cells. TEBs are the least differentiated and most actively growing glandular ductal structures, which are most abundant from weaning to puberty and are the site of chemically induced tumors (Ip *et al.*, 2003).

#### 17.6.1.2. Studies with Rumenic Acid

The early studies with CLA used a synthetic mixture of many isomers, but usually contained about 70–80% of near equal quantities of RA and *trans*-10, *cis*-12-C<sub>18:2</sub>. Recently, a number of studies have demonstrated that both isomers can inhibit mammary tumor development (Ip *et al.*, 2003).

In a novel experiment, Ip *et al.* (1999) demonstrated that rats fed a RA-enriched butter had reduced mammary epithelial mass, decreased size of the TEB population, suppressed proliferation of TEB cells, and inhibition of mammary tumor incidence and tumor number similar to animals fed an equivalent quantity of mixed CLA isomers. There was a consistently higher level of RA in the liver, mammary fat pad, peritoneal fat, and blood plasma of rats fed RA-enriched butter compared to CLA-fed rats. The elevated RA levels resulted, no doubt, from endogenous  $\Delta^9$ -desaturation of VA, which was concurrently increased in the RA-enriched butter.

A follow-up study demonstrated that pure isomers of RA and *trans*-10, *cis*-12-C<sub>18:2</sub> were equivalent in preventing the development of both MNU-induced premalignant lesions, called intraductal proliferations (IDPs) and tumors in the mammary gland (Ip *et al.*, 2003). Later, it was shown that feeding rats a diet containing 2% VA inhibited MNU-induced IDPs to the same degree as feeding a diet containing 1% RA (Banni *et al.*, 2001).

### 17.6.1.3. Mechanisms for the Anti-Tumor Action of RA

Tumors grow when the rate of cell proliferation exceeds apoptosis. Apoptosis is the mechanism of programmed cell death by which the body eliminates unwanted or damaged cells. At the molecular level, a number of mechanisms by which RA exerts its antitumor effects have been demonstrated. RA, like mixed CLA isomers, inhibits the proliferation of malignant mammary tumor cells. In addition, RA inhibits proliferation in normal mammary epithelial cells. This reduction in proliferation was associated with decreased expression of the cell regulatory biomarkers, cyclin D1 and cyclin A. However, rapidly proliferating cells in young rats were more sensitive to RA treatment than more quiescent cells in older animals. When rats were fed RA, apoptosis was induced in MNU-induced IDPs, but not in normal TEBs or more differentiated alveoli structures. The induction of apoptosis in IDPs was associated with reduced expression of the anti-apoptotic regulatory protein bcl-2 (Ip *et al.*, 2003).

Angiogenesis is the process of forming new blood vessels, which are necessary for the growth and spread of tumors. Masso-Welch *et al.* (2004) demonstrated that RA effectively inhibits angiogenesis in mice. This inhibition was related to reduced serum levels of vascular endothelial growth factor (VEGF). VEGF not only plays a central role in promoting angiogenesis, but can also stimulate the growth and invasiveness of breast cancer cells (Ip *et al.* 2003). Hubbard *et al.* (2003) reported that RA reduced pulmonary tumor metastasis in mice when mammary tumor cells were either transplanted into mammary fat pads or injected intravenously *via* the tail vein.

Two other, as yet largely unproven, mechanisms for the anti-tumorigenic action of RA are the reduction in COX-induced pro-tumorigenic eicosanoid species, and the activation of PPARs. RA may displace linoleic acid or its metabolite, arachidonic acid, from membrane phospholipids and from neutral lipids. This decreases available substrate for the tumor-promoting prostacyclins, thromboxanes and prostaglandins, generated by constitutive COX-1 and the inducible form COX-2. It is also possible that RA, or its metabolites, can directly inhibit COX activity (Belury, 2002; Parodi 2004). As discussed previously, RA can bind and activate PPAR $\gamma$ . Moreover, RA can also increase the expression of PPAR $\gamma$  (Brown *et al.*, 2003). Synthetic PPAR $\gamma$  agonists have been shown to protect against cancer at a number of sites including the breast (Desvergne and Wahli, 1999; Kersten *et al.*, 2000).

### 17.6.1.4. Epidemiology

An initial case-control study on Finnish women found an inverse association between RA and breast cancer in post-menopausal women, but

not in pre-menopausal women (Aro *et al.*, 2000). After adjusting for known risk factors, post-menopausal women in the lowest quintile of dietary RA had a threefold greater risk of breast cancer compared to women in the highest quintile of intake. Similarly, women in the lowest quintiles of plasma RA and VA levels both had a fivefold greater risk of breast cancer.

Since then, three other epidemiological studies have failed to confirm a protective role for RA or VA in the development of breast cancer. A Dutch prospective study used dietary assessment, a French study compared RA levels in tissue adjacent to malignant tumors with levels adjacent to benign tumors, and the third study was a nested case-control study of Finnish women using fatty acid data from plasma collected prospectively. Recently, a fourth study showed that, although higher intakes of CLA were not related to overall breast cancer risk, risk of estrogen receptor negative breast cancer—the most aggressive form—in pre-menopausal women was reduced (Mc Cann *et al.*, 2004). Parodi (2004) has described methodological inadequacies in these studies.

### 17.6.2. Sphingolipids and Colon Cancer

Evidence suggests that dietary sphingolipids, acting through their hydrolysis products, ceramide and sphingosine, can inhibit the development of colon cancer (Duan, 1998; Vesper *et al.*, 1999). In milk fat, sphingomyelin is by far the predominant sphingolipid.

#### 17.6.2.1. Mechanisms: the Sphingomyelin Signalling Pathway

Sphingomyelin is concentrated in the outer leaflet of cellular membranes and its function was thought to be solely associated with structural stability. However, an important sphingomyelin-signalling pathway has been established recently. Extracellular agonists such as TNF- $\alpha$ , interferon  $\gamma$ , IL-1, and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) bind and activate their cellular receptors to stimulate the release of sphingomyelinase. The liberated sphingomyelinase cleaves sphingomyelin to produce ceramide. In turn, ceramide acts as a second messenger for the extracellular agonists, transmitting the signal through multiple downstream targets, such as protein phosphatases and protein kinases. These targets, in turn, regulate a number of transcription factors that control the expression of a range of genes responsible for inhibition of cell growth, cell cycle arrest, differentiation, and apoptosis.

Other agonists, like platelet-derived growth factor, can induce ceramidase, which hydrolyses ceramides to sphingosine. Sphingosine also acts as a second messenger in signalling cascades that regulate cell growth. Because of their biological activity in regulating cell growth, ceramide and

sphingosine are referred to as tumor suppressor lipids (Duan, 1998; Vesper *et al.*, 1999).

#### 17.6.2.2. Digestion and Tissue Enhancement

Unlike other dietary lipids, sphingolipids are poorly digested in the proximal small intestine, and digestion continues slowly in the distal small intestine and in the colon. The rate of sphingolipid digestion in the intestinal tract mirrors the distribution pattern for alkaline sphingomyelinase in the intestine. Studies in rats found that the digestion products of sphingolipids, mainly ceramides and sphingosine, are absorbed rapidly by intestinal cells where they can be degraded further or re-incorporated into sphingolipids that mostly remain associated with the intestine. Nevertheless, some labelled sphingoid base has been detected in lymph, plasma and the liver (Nyberg *et al.*, 1997; Duan, 1998; Vesper *et al.*, 1999). Nyberg *et al.* (1997) found that the ceramide content in the colonic mucosa increased with increasing sphingomyelin intake. Enhancement of colonic tissue with ceramide, as a result of dietary sphingolipid intake, could be important for the prevention of colon cancer.

#### 17.6.2.3. Sphingomyelinase, Ceramide and Human Carcinomas

The importance of alkaline sphingomyelinase and ceramides in colon cancer is suggested by studies that compared their levels in tumors with levels in normal colonic tissue. Hertervig *et al.* (1997) found that the activity of alkaline sphingomyelinase was 75% less in tissue from human colon carcinomas than in normal colonic tissue. In familial adenomatous polyposis patients, there was a 90% decrease in alkaline sphingomyelinase activity in colonic adenomas and in surrounding mucosa compared to healthy controls (Hertervig *et al.*, 1999). Human colon carcinomas had a 50% decrease in cellular ceramide content compared to normal colon mucosa (Selzner *et al.*, 2001).

#### 17.6.2.4. Cell Culture Studies

Ceramides and sphingosine can inhibit cell growth, promote differentiation and induce apoptosis in a range of human cancer cell lines, including several types of colon cancer cells (Parodi, 2004). Selzner *et al.* (2001) established that ceramides induced cell death in cultured human SW403 colon cancer cells. Treatment of these cells with a ceramidase inhibitor, which prevented the catabolism of ceramides and thus increased its content in the cells, resulted in suppressed cell growth and induction of apoptosis. Selzner *et al.* (2001) then injected SW403 cancer cells and

human metastatic Lovo cancer cells into the portal vein of mice. Administration of a ceramidase inhibitor to these mice inhibited colon cancer metastases to the liver.

Bile acids that escape enterohepatic circulation and pass to the colon can be cytotoxic to colonocytes. Damaged cells undergo apoptosis and are shed into the lumen. To maintain cell homeostasis, new cells must be produced. This replacement can result in an increase in cell proliferation rate that can increase the risk of mutations in tumor-related genes and lead to carcinoma development. Moschetta *et al.* (2000) showed that sphingomyelin protected against bile acid-induced cytotoxicity in human CaCo-2 colon cancer cells, a common model for studying intestinal cell function.

#### 17.6.2.5. Animal Tumor Studies

A series of studies show that sphingolipids derived from milk protect against development of colon tumors in mice. In the initial study (Dillehay *et al.*, 1994), mice fed diets supplemented with 0.025, 0.050 or 0.1% sphingomyelin had less than half the incidence of 1,2-dimethylhydrazine (DMH)-induced colon tumors than mice fed non-supplemented diets. In addition, a diet supplemented with 0.025% of the ganglioside GM<sub>1</sub> produced significantly fewer DMH-induced aberrant crypt foci (ACF) than control animals. ACF are microscopically determined pre-neoplastic lesions that can develop into colon tumors.

In a follow-up study (Schmelz *et al.*, 1996), mice were fed 0–0.1% sphingomyelin isolated from low-fat milk or buttermilk. Sphingomyelin from both sources reduced DMH-induced ACF by 70%. In a longer-term study, sphingomyelin had no effect on the incidence or multiplicity of chemically induced colon tumors. However, up to 31% of tumors in the mice fed sphingomyelin were adenomas, whereas all of the tumors in the non-supplemented mice were malignant adenocarcinomas. In order to eliminate the possibility that an unknown component isolated from milk along with sphingomyelin was the anti-tumor agent, Schmelz *et al.* (1997) compared the action of milk-derived sphingomyelin with a synthetically prepared product. Both sources of sphingomyelin reduced DMH-induced ACF formation to the same extent.

Schmelz *et al.* (2000) then isolated the complex sphingolipids, glucosylceramide, lactosylceramide and the ganglioside GD<sub>3</sub>, from milk. These sphingolipids were added individually to the diet of mice at a level of 0.025 or 0.1%. All three sphingolipids reduced the number of DMH-induced ACF by about 40%, a reduction that was comparable to that obtained by sphingomyelin in earlier experiments.

The next study (Schmelz *et al.*, 2001), used a different model, the *Apc*<sup>Min/+</sup> mouse. In this model the mice have a germline mutation in the adenomatous polyposis coli (*Apc*) gene and they spontaneously develop adenomas (polyps) throughout the intestinal tract, but preferentially in the small intestine. In humans, germline mutations in the *APC* gene (a tumor suppressor gene) are responsible for familial adenomatous polyposis, a syndrome in which patients develop multiple benign adenomas in the colon. If not resected, a proportion of these adenomas will progress to become malignant. In sporadic colon cancer, mutations in the *APC* gene initiate the development of most tumors.

*Apc*<sup>Min/+</sup> mice were fed a control diet or that diet supplemented with either 0.1% ceramide, a sphingolipid mixture of sphingomyelin, glucosylceramide, lactosylceramide and ganglioside GD<sub>3</sub> with a composition similar to that found in dairy products, or this mixture plus ceramide (60:40). All three diets significantly reduced the number of spontaneously developed tumors in all regions of the intestines (Schmelz *et al.*, 2001).

In the preceding experiments, sphingolipid supplementation commenced after tumor initiation with a chemical carcinogen. Lemonnier *et al.* (2003) fed 0.05% sphingomyelin to a group of mice before tumor initiation and to another group after tumor initiation. Both dietary protocols drastically reduced tumor formation to the same extent, which suggests that sphingolipids may have both chemopreventive and chemotherapeutic benefits. In this study, tumor inhibition was associated with normalization of cell proliferation and the rate of apoptosis.

This is an exciting area of research and further mechanistic studies leading to human intervention studies must follow.

### 17.6.3. Butyric Acid

Uniquely, milk fat of ruminants contains butyric acid, which is an important anti-cancer agent. Butyric acid is best known for its action in the colon where it is generated, along with other short-chain acids, by bacterial fermentation of dietary fiber and starch. Colonocytes utilize a portion of this butyric acid as a primary energy source, with the remainder delivered to the portal circulation and transported to the liver where it is metabolized rapidly.

A number of animal studies have shown that dietary fibers, which liberate a constant and elevated supply of butyrate to the colon, are the most effective for prevention of chemically induced colon tumors. Moreover, the level of butyric acid in the colonic lumen of patients with colorectal cancer and adenomas was found to be lower than that in healthy individuals (Parodi, 2004).

### 17.6.3.1. Mechanistic Studies

Low concentrations of butyric acid can inhibit growth in a wide range of human cancer cell lines, including prostate and several types of breast and colon cancer by a number of mechanisms (Williams *et al.*, 2003; Parodi, 2004). It is believed that central to the anti-cancer action of butyric acid is its ability to inhibit histone deacetylases, which results in histone hyperacetylation and destabilization of chromatin structure that facilitates transcription factor binding and activation of genes associated with cell growth. Histone hyperacetylation activates the *p21* gene, which inhibits the cell cycle regulators, cyclin D1 and cyclin B1. This results in the inhibition of cyclin-dependent kinase, which in turn prevents phosphorylation of retinoblastoma protein required for progress from the pre-synthetic G<sub>1</sub> phase of the cell cycle to the S phase. The arrested cells may then differentiate or undergo apoptosis (Davie, 2003). A summary of these related cell-growth inhibiting mechanisms induced by butyric acid (Williams *et al.*, 2003; Parodi, 2004) are outlined below:

- Inhibition of proliferation.
- Induction of terminal differentiation.
- Induction of apoptosis associated with increased caspase (cysteine protease) activity and decreased expression of the anti-apoptotic Bcl-2 protein along with increased expression of the pro-apoptotic Bak and Bax proteins
- Inhibition of angiogenesis associated with down-regulation of VEGF.
- Anti-inflammatory action. Chronic or recurrent inflammation probably has a role in many types of human cancer.
- Up-regulation of immunosurveillance.
- Increased expression of insulin-like growth factor binding protein-3 (IGFBP-3), the major plasma binding protein for insulin like growth factor-1 (IGF-1). Elevated levels of IGF-1 relative to levels of IGFBP-3 are implicated in the development of several human cancers, including colon, breast and prostate.
- Enhanced expression of glutathione *S*-transferase (GST). GSTs inactivate carcinogens by catalyzing their conjugation to glutathione, forming water-soluble metabolites that are easily excreted.
- Suppression of colonocyte NF- $\kappa$ B activation (Yin *et al.*, 2001). NF- $\kappa$ B is a transcription factor that regulates several signalling patterns involved in cell proliferation and apoptosis. In many tumors activation of NF- $\kappa$ B promotes proliferation and inhibits apoptosis. Activation of NF- $\kappa$ B is also associated with inflammatory response.

### 17.6.3.2. Butyric Acid as a Chemotherapeutic Agent

Early attempts to use butyrate in the treatment of human cancer—usually in patients refractory to other treatments—were unsuccessful (Pouillart, 1998). Failure was largely due to the very short half-life of butyrate in the circulation. The appeal of butyrate as a therapeutic agent lies in the absence of systemic toxicity, therefore a series of butyrate analogues—referred to as prodrugs—that increase the plasma half-life of butyrate were developed. One simple prodrug is the triacylglycerol, tributyrin. Orally administered tributyrin increased and extended plasma butyrate concentration in rats and mice (Egorin *et al.*, 1999) and in humans (Conley *et al.*, 1998). Thus, enhanced plasma butyrate levels may allow anti-cancer action at sites other than the colon. In milk fat, one triacylglycerol molecule in three contains butyrate (Parodi, 2004).

### 17.6.3.3. Mammary Tumor Prevention

Two studies have shown that dietary butyrate inhibits chemically-induced mammary tumor development in rats. First, Yanagi *et al.* (1993) showed that addition of 6% sodium butyrate to a basal diet containing 20% fat, supplied by a margarine made from safflower oil, significantly reduced the incidence of DMBA-induced mammary carcinomas and adenocarcinomas. In the second study, Belobrajdic and McIntosh (2000) fed rats a 20% fat diet consisting of either milk fat, sunflower seed oil (SSO), SSO + 1% tributyrin (butyric acid content equivalent to milk fat) or SSO + 3% tributyrin. At any period during the course of the experiment, there was a relative risk increase of 88% that rats consuming the SSO diet would develop MNU-induced mammary tumors compared to those in the milk fat group. The addition of 1% or 3% tributyrin to the SSO diets reduced tumor incidence by 20 and 52%, respectively, in comparison to SSO alone.

### 17.6.3.4. Synergy

There are a number of studies that show synergism between butyrate and other dietary components and common drugs in reducing cancer cell growth. This could result in lower plasma butyrate requirements for anti-cancer action (Parodi, 2004). In summary:

- Retinoic acid, at concentrations likely to be found in plasma, reduced by tenfold the level of butyric acid required to induce differentiation.
- Physiological concentrations of  $1, 25(\text{OH})_2\text{D}_3$  acted synergistically with butyrate to inhibit proliferation and induce differentiation.

- Resveratrol, a plant polyphenol found in red wine and grapes, enhanced the ability of butyrate to induce differentiation.
- A HMG CoA reductase inhibitor, used for treatment of hypercholesterolemia, enhanced butyrate-induced inhibition of proliferation.
- Aspirin, now commonly used for prevention of CHD and colon cancer, combined with butyrate exerted stronger anti-proliferate and pro-apoptotic effects than either alone.

#### 17.6.4. 13-Methyltetradecanoic Acid

13-Methyltetradecanoic acid (13-MTDA) is a 15-carbon fatty acid with a terminal isopropyl group. It is synthesized by bacteria in the rumen, along with other branched-chain fatty acids. Yang *et al.* (2000) demonstrated that low concentrations of 13-MTDA could induce cell death in a range of human cancer cell lines. The cell lines tested were prostate, colon, lung (small cell), liver, and gastric carcinomas, mammary and pancreatic adenocarcinomas, and leukemia. 13-MTDA-initiated cell death resulted from rapid induction of apoptosis.

Human lung cancer cells and human prostate cancer cells were implanted into athymic nude mice, then harvested and implanted in the lung and prostate, respectively, of a MetaMouse orthotopic model. Feeding 13-MTDA for 40 days inhibited the growth of lung cancer implants by 65% and prostate cancer implants by 85% compared to control animals (Yang *et al.*, 2000).

Recently, Wongtangintharn *et al.* (2004) showed that a series of *iso*- and *anteiso*-branched-chain fatty acids was cytotoxic to breast cancer cells. The highest activity was observed with *iso*-C<sub>16:0</sub> and the activity decreased as the chain-length increased or decreased from C<sub>16:0</sub>. Cytotoxicity of branched-chain fatty acids was comparable to that of RA and *trans*-10, *cis*-12-C<sub>18:2</sub>.

#### 17.6.5. Ether Lipids

Milk fat contains small amounts of ether lipids. Synthetic ether lipids at low concentrations are potent anti-neoplastic agents. Cell culture studies showed that they inhibited cell growth, induced differentiation, promoted apoptosis and showed anti-metastatic activity. In human chemoprevention trials ether lipids have been administered parenterally. Although the ether bond is preserved when these lipids are delivered orally, no cancer chemoprevention studies have utilized dietary ether lipids (Parodi, 2004).

#### 17.6.6. Cholesterol

Evidence of a role for dietary cholesterol in carcinogenesis, using animal models, is contradictory. This may result, in part, from contamination of

the test cholesterol with oxidation products that are carcinogenic and the use of supra-physiological doses. Nevertheless, dietary cholesterol was shown to inhibit chemically-induced colon tumors (El-Sohemy et al., 1996a) and mammary tumors (El-Sohemy et al., 1996b) in serum-cholesterol sensitive rats. The researchers consider that the accompanying elevated serum cholesterol levels triggered the inhibition of endogenous cholesterol synthesis by reducing the level of HMG-CoA reductase. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway that converts HMG-CoA to mevalonate, which is required for DNA synthesis during rapid cell proliferation associated with the early stage of tumorigenesis.

### 17.6.7. $\beta$ -Carotene and Vitamin A

Milk fat supplies the diet with a substantial proportion of its daily  $\beta$ -carotene and vitamin A requirements. Dietary  $\beta$ -carotene is converted to retinal in the intestinal epithelium and in the liver by the enzyme  $\beta$ -carotene-15–15'-dioxygenase. The retinal formed is further metabolized to retinoic acid (vitamin A).

Epidemiological evidence suggests that diets rich in  $\beta$ -carotene—largely fruit and vegetables—and high plasma levels of  $\beta$ -carotene are inversely associated with the risk of cancer, especially esophagus, lung, stomach, colorectal, breast and cervical cancers (Cooper, 2004).

Both  $\beta$ -carotene and vitamin A can inhibit growth in a large range of human cancer cell lines (Krinsky, 1993; Niles, 2000). Several animal studies have shown that vitamin A deficiency promotes the development of spontaneous and chemically-induced tumors, whereas dietary supplementation with vitamin A can prevent chemically-induced tumor development (Niles, 2000). Likewise,  $\beta$ -carotene protects against tumor development in animal models (Krinsky, 1993; Cooper, 2004; Russell, 2004). Nevertheless,  $\beta$ -carotene is preferred for human studies because blood and tissue levels increase in proportion to dietary intake, whereas vitamin A level does not increase in a linear manner because of homeostatic regulation; high levels of vitamin A are toxic (Cooper, 2004).

There has been some success with vitamin A and its derivatives for treatment of certain types of cancer (Niles, 2000). However, the use of  $\beta$ -carotene as a therapeutic agent suffered a setback when the results from two of three large human intervention studies indicated that high doses of  $\beta$ -carotene caused an increased risk of lung cancer in smokers and subjects exposed to asbestos. This increased risk is thought to be due to metabolites associated with high doses of  $\beta$ -carotene in the presence of smoke (Russell, 2004).

### 17.6.8. Vitamin D and its Metabolites

Milk fat is not a rich source of vitamin D, but in some countries dairy products are fortified with this vitamin. Vitamin D, through its metabolites, is an anti-cancer agent of increasing importance. Vitamin D results from ultraviolet light-catalyzed conversion of 7-dehydrocholesterol in the skin. The pre-vitamin D<sub>3</sub> thus formed is transported to the liver, where it is converted to 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>], which is the main circulating form of the vitamin. The circulating 25(OH)D<sub>3</sub> is subsequently converted to 1, 25(OH)<sub>2</sub>D<sub>3</sub> in the kidneys by the enzyme 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase. This conversion is regulated by physiological requirements (Hansen *et al.*, 2001; Zitterman, 2003; Holick, 2004). It is now realized that a number of tissues, including colon, breast and lung contain 1 $\alpha$ -hydroxylase and can produce 1, 25(OH)<sub>2</sub>D<sub>3</sub>, which acts in an autocrine manner to regulate cell growth (Holick, 2004).

1, 25(OH)<sub>2</sub>D<sub>3</sub> inhibited proliferation and induced differentiation and apoptosis in human colon, breast, prostate and gynecological cancers as well as several forms of hematopoietic cancer (Studzinski and Moore, 1995; van Leeuwen and Pols, 1997). Experimental animal studies show that 1, 25(OH)<sub>2</sub>D<sub>3</sub> inhibited chemically-induced breast, colon, and skin tumors. The growth of colon, breast and prostate cancer cells, as well as melanoma and retinoblastoma cells implanted into rodents was retarded by treatment with 1, 25(OH)<sub>2</sub>D<sub>3</sub> (Studzinski and Moore, 1995; van den Bemd *et al.*, 2000).

Epidemiologic evidence suggests that low exposure to sunlight, low dietary intake of vitamin D and low plasma levels of 25(OH)D<sub>3</sub> and 1, 25(OH)<sub>2</sub>D<sub>3</sub> increase the risk of developing colon, breast and prostate cancer (Studzinski and Moore, 1995; van den Bemd *et al.*, 2000; Zittermann, 2003). There is evidence that vitamin D deficiency can attenuate the beneficial effect of dietary calcium for the prevention of colonic adenoma and carcinoma (Parodi, 2001a).

### 17.6.9. Anti-Cancer Agents from Feed

The cow has a remarkable capability to extract biological components from its feed and transfer them to its milk. The best-known example is  $\beta$ -carotene from pasture, a portion of which is converted to vitamin A *in vivo*, so that milk fat contains both these anti-cancer compounds. Vitamin E can also be obtained from the cow's feed. Cows fed cottonseed meal transfer the polyphenol, gossypol, to milk, and alfalfa or lucerne provides  $\beta$ -ionone. Both gossypol and  $\beta$ -ionone, an HMG-CoA reductase inhibitor, are demonstrated anti-cancer agents (Parodi, 2004).

Milk fat contains small quantities of phytanic and pristanic acid. Phytanic acid is produced by bacterial cleavage of the phytol side chain of plant chlorophyll in the rumen. Some phytanic acid is converted to pristanic acid by  $\alpha$ -oxidation in the liver. Both of these branched-chain acids are agonists for PPAR $\alpha$  at physiological concentrations (Parodi, 2004). Milk fat from cows fed cannery fruit and vegetable waste no doubt contains other interesting phytochemicals with anti-cancer potential.

#### 17.6.10. Milk Fat and Cancer

Milk fat contains a number of components with anti-cancer potential, but many of these components are present at levels lower than those shown to produce a benefit in *in vitro* and *in vivo* experimental models. Nevertheless, as outlined in Section 17.6.3, synergy between anti-cancer components in milk fat and with other components from dietary items can lower several fold the concentration required to produce a physiological effect.

A role for milk fat in cancer risk has not been examined adequately in epidemiological studies, because milk fat is not consumed as a single dietary item, but as a component of dairy products and dairy products also contain non-lipid components with anti-cancer potential (Parodi, 2001a, b). On the other hand, seven studies were found in the literature, where milk fat or butter diets were compared with diets containing equal amounts of polyunsaturated vegetable oils or margarine in animal models of colon, breast, and skin cancer. All seven studies showed that there was less tumor development with milk fat-based diets (Parodi, 2004).

#### 17.7. Other Nutritional Benefits

When milk fat is included in the diet it can confer a number of additional health related benefits. These aspects of milk fat nutrition have recently been reviewed in some detail (Parodi, 2004) and are summarized briefly below:

- Children fed low-fat milk had up to a fivefold greater incidence of acute gastrointestinal illness than children who were fed whole milk.
- Studies *in vitro* and in rats showed that short and medium chain fatty acids and monoacylglycerols hydrolyzed from milk triacylglycerols and digestion products of sphingolipids possess strong anti-bacterial and anti-viral properties.
- Milk fat is not a rich source of linoleic ( $\omega$ -6) and linolenic ( $\omega$ -3) acids; however, the ratio of  $\omega$ -6:  $\omega$ -3 is close to unity, which is considered to be ideal for good health. Rats fed diets high in milk fat had a beneficial long-chain polyunsaturated fatty acid profile in plasma

and aortic phospholipids, where arachidonic acid levels were reduced and the levels of eicosapentaenoic and docosahexaenoic acids enhanced. An equivalent benefit was not observed when rats were fed the same level of vegetable oils or lard.

- Milk phospholipids were shown to protect against stress, bacterial, and chemical-induced gastric mucosal damage.
- Milk fat may promote bone formation.
- There was less plaque formation on human tooth surfaces in subjects who drank whole milk than in subjects who drank skim milk or water.
- There is accumulating evidence that consumption of whole milk compared to skim milk and the consumption of butter compared to margarine is associated with a lower incidence of asthma and other allergic disorders.

## 17.8. Conclusions

The demonization of fat during the past two decades is unwarranted. There is no compelling evidence that fat is responsible for the current obesity epidemic or is implicated in weight gain independent of energy density. Evidence from well-conducted animal and epidemiological studies does not support a role for fat in the etiology of breast, prostate and colon cancer, the major non-smoking-related cancers.

Even though milk fat contains some fatty acids that may elevate plasma total and LDL-cholesterol levels, which are risk factors for CHD, this effect is balanced by concurrent increases in levels of anti-atherogenic HDL-cholesterol. In addition, saturated fatty acids reduce plasma levels of atherogenic Lp[a] and produce a less atherogenic LDL particle size. Dietary intervention studies, where there was a substantial reduction in saturated fat intake and plasma cholesterol levels, did not produce an improvement in CHD or total mortality. Prospective epidemiological studies provide no evidence that saturated fatty acids are a risk factor for CHD. Indeed, in two large studies, saturated fatty acids were inversely associated with risk.

Milk fat contains several compounds that have demonstrated anti-cancer activity in animal models. The more important ones are ruminic acid, a potent inhibitor of mammary tumorigenesis, sphingomyelin and other sphingolipids that prevent the development of intestinal tumors and butyric acid, which prevents colon and mammary tumor development. Emerging evidence suggests that milk fat can prevent intestinal infections, particularly in children, prevent allergic disorders, such as asthma and improve the level of long-chain  $\omega$ -3 polyunsaturated fatty acids in blood.

Fat is an essential component of the diet, and inclusion of milk fat as part of a balanced diet should be advantageous rather than detrimental.

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# Oxysterols: Formation and Biological Function

P.A. Morrissey and M. Kiely

## 18.1. Introduction

The association between plasma cholesterol (CHOL) and atherosclerosis has been a topic of research for many decades and continues to be a major field of investigation (McNamara, 2000; Kromhout, 2001). A considerable number of studies, both in animals and humans, have provided evidence that prolonged high levels of plasma CHOL increase the risk of developing atherosclerosis. However, the mechanisms by which CHOL contributes to the initiation and progression of atherosclerosis are still the subject of intense research. The observation that oxidized low-density lipoprotein (LDL) triggers early steps in atherogenesis (Steinberg *et al.*, 1989; Ross, 1993) has focused attention on the role(s) of oxidizable components of LDL [namely the polyunsaturated fatty acid (PUFA) content], and the involvement of antioxidant vitamins, such as vitamins C and E and the carotenoids (especially  $\beta$ -carotene) in the prevention of cardiovascular diseases (Gey *et al.*, 1991, 1993; Kushi *et al.*, 1996). A number of compounds have been identified in oxidatively-modified LDL that elicit the development of atherosclerosis (Ross, 1999) and fatty streak formation (Lusis, 2000). In several cases, the compounds arise from oxidized lipids, including CHOL oxidation products or oxysterols (OS). When LDL is oxidized, the associated PUFA content is reduced, and the levels of lipid peroxides, aldehydes and OS increase (Patel *et al.*, 1996; Chang *et al.*, 1997). Several OS are of interest as possible reactive mediators of the structural and functional changes of the vascular system that are characteristic of the atherosclerotic

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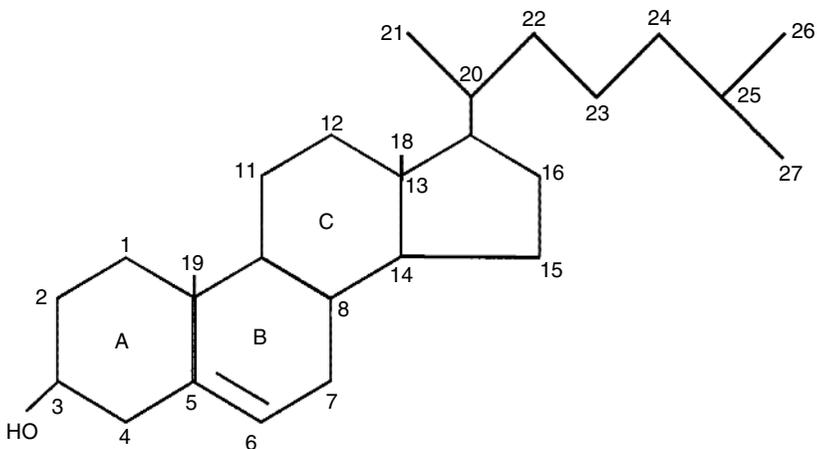
*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

process (Sevanian *et al.*, 1995; Smith, 1996; Guardiola *et al.*, 1996; Brown and Jessup, 1999; Russell, 2000; Schroepfer, 2000; Leonarduzzi *et al.*, 2002). OS may also be linked to a wide range of other biological effects in humans and animals, including cytotoxicity, mutagenesis, carcinogenesis, necrosis, apoptosis, immunosuppression and the development of gallstones (Brown and Jessup, 1999; Lyons and Brown, 1999; Lizard *et al.*, 2000; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). On the other hand, OS may regulate the expression of genes that participate in the metabolism of both sterols and fats, are intermediates in the transfer of sterols from the periphery to the liver and serve as substrates for the synthesis of bile acids (Russell, 2000). However, the mechanistic details and the identity of some of the native OS species have not been established with certainty.

## 18.2. Formation of Oxysterols

Oxysterols are defined as oxygenated derivatives of cholest-5-en-3 $\beta$ -ol (cholesterol) (Figure 18.1) or precursors of CHOL that may be formed directly by autoxidation or by the action of a specific monooxygenase, or that may be secondary to enzymatic or nonenzymatic lipid peroxidation (Guardiola *et al.*, 1996; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). These OS may be formed in the human body by endogenous free-radical attack on CHOL or by enzymatic processes, mainly in the biosynthesis of bile acids and steroid hormones. In addition, OS may be formed exogenously by autoxidation of CHOL in foods. The nomenclature and abbreviations of OS are presented in Table 18.1. It should be emphasized at this point that the OS that occur in



**Figure 18.1.** The cholest-5-en-3 $\beta$ -ol (cholesterol) molecule, showing ring labelling and carbon numbering.

**Table 18.1.** Nomenclature and Abbreviations of Cholesterol Oxidation Products

Systematic name	Trivial name	Abbreviation
Cholest-5-en-3 $\beta$ -ol	Cholesterol	CHOL
3 $\beta$ -Hydroxycholest-5-en-7-yl	Cholesterol 7-radical	7-CHOL <sup>•</sup>
3 $\beta$ -Hydroxycholest-5-en-7-peroxyl radical	Cholesterol 7-peroxyl radical	CHOO <sup>•</sup>
3 $\beta$ -Hydroxycholest-5-en-7-radical	Cholesterol 7-alkoxyl radical	CHO <sup>•</sup>
3 $\beta$ -Hydroxycholest-5-ene-7 $\alpha$ -hydroperoxide	7 $\alpha$ -Hydroperoxycholesterol	7 $\alpha$ -OOH
3 $\beta$ -Hydroxycholest-5-ene-7 $\beta$ -hydroperoxide	7 $\beta$ -Hydroperoxycholesterol	7 $\beta$ -OOH
3 $\beta$ -Hydroxycholest-6-ene-5 $\alpha$ -hydroperoxide	5 $\alpha$ -Hydroperoxycholesterol	5 $\alpha$ -OOH
3 $\beta$ -Hydroxycholest-4-ene-6 $\alpha$ -hydroperoxide	6 $\alpha$ -Hydroperoxycholesterol	6 $\alpha$ -OOH
3 $\beta$ -Hydroxycholest-4-ene-6 $\beta$ -hydroperoxide	6 $\beta$ -Hydroperoxycholesterol	6 $\beta$ -OOH
3 $\beta$ -Hydroxycholest-5-ene-25-hydroperoxide	25-Hydroperoxycholesterol	25-OOH
3 $\beta$ -Hydroxycholesterol-5-ene-20 $\alpha$ -hydroperoxide	20 $\alpha$ -Hydroperoxycholesterol	20 $\alpha$ -OOH
Cholest-5-ene-3 $\beta$ ,7 $\alpha$ -diol	7 $\alpha$ -Hydroxycholesterol	7 $\alpha$ -OH
Cholest-5-ene-3 $\beta$ ,7 $\beta$ -diol	7 $\beta$ -Hydroxycholesterol	7 $\beta$ -OH
Cholest-6-ene-3 $\beta$ ,5 $\alpha$ -diol	5 $\alpha$ -Hydroxycholesterol	5 $\alpha$ -OH
Cholest-4-ene-3 $\beta$ ,6 $\alpha$ -diol	6 $\alpha$ -Hydroxycholesterol	6 $\alpha$ -OH
Cholest-4-ene-3 $\beta$ ,6 $\beta$ -diol	6 $\beta$ -Hydroxycholesterol	6 $\beta$ -OH
Cholest-5-ene-3 $\beta$ ,4 $\alpha$ -diol	4 $\alpha$ -Hydroxycholesterol	4 $\alpha$ -OH
Cholest-5-ene-3 $\beta$ ,4 $\beta$ -diol	4 $\beta$ -Hydroxycholesterol	4 $\beta$ -OH
Cholest-5-ene-3 $\beta$ ,25-diol	25-Hydroxycholesterol	25-OH
Cholest-5-ene-3 $\beta$ ,20 $\alpha$ -diol	20 $\alpha$ -Hydroxycholesterol	20 $\alpha$ -OH
3 $\beta$ -Hydroxycholest-5-en-7-one	7-Ketocholesterol	7-keto
3 $\beta$ -Hydroxycholest-4-en-6-one	6-Ketocholesterol	6-keto
5 $\alpha$ -Cholestane-3 $\beta$ ,5,6 $\beta$ -triol	Cholestanetriol	Triol
5,6 $\alpha$ -Epoxy-5 $\alpha$ -cholestan-3 $\beta$ -ol	Cholesterol 5 $\alpha$ ,6 $\alpha$ -epoxide	$\alpha$ -epoxide
5,6 $\beta$ -Epoxy-5 $\beta$ -cholestan-3 $\beta$ -ol	Cholesterol 5 $\beta$ ,6 $\beta$ -epoxide	$\beta$ -epoxide
Cholesta-3,5-diene		3,5-diene
Cholesta-3,5-dien-7-one		7-keto-3,5-dien
Cholesta-3,5,7-triene		3,5,7-triene
Cholest-5-en-3-one	5-Cholesten-3-one	3-keto-5-en
Cholest-4-en-3-one	4-Cholesten-3-one	3-keto-4-en
Cholesta-5,7-dien-3 $\beta$ -ol		5,7-dien-3-ol

biological membranes are normally present in trace amounts and always with a great excess ( $10^3$ - to  $10^6$ -fold) of CHOL.

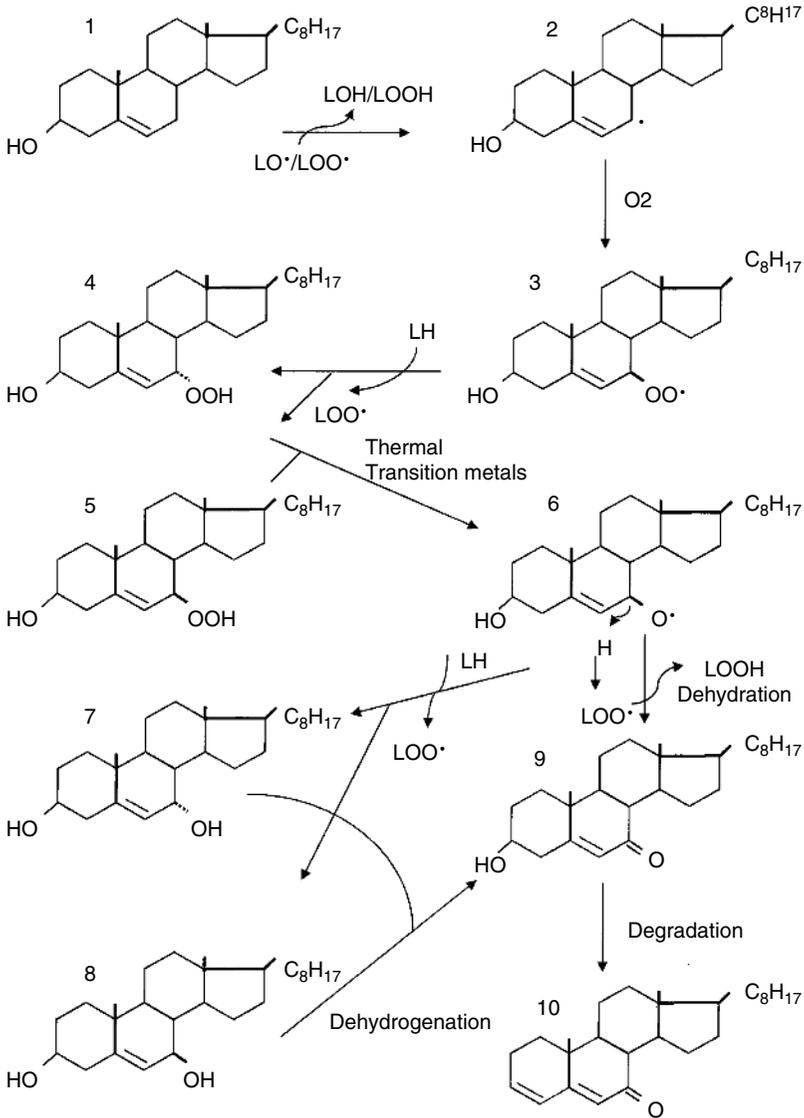
### 18.2.1. Cholesterol Autoxidation

Early research on the oxidation of CHOL has been reviewed extensively and evaluated by Smith (1987, 1992, 1996), Schoepfer (2000) and Lercker and Rodriguez-Estrada (2002). CHOL, with its double bond between the C-5 and C-6 of the B-ring (Figure 18.1), readily undergoes oxidation *via* a free radical mechanism to form  $\sim 75$  OS products (Smith, 1996). Among the potential initiators of CHOL oxidation are preformed lipid

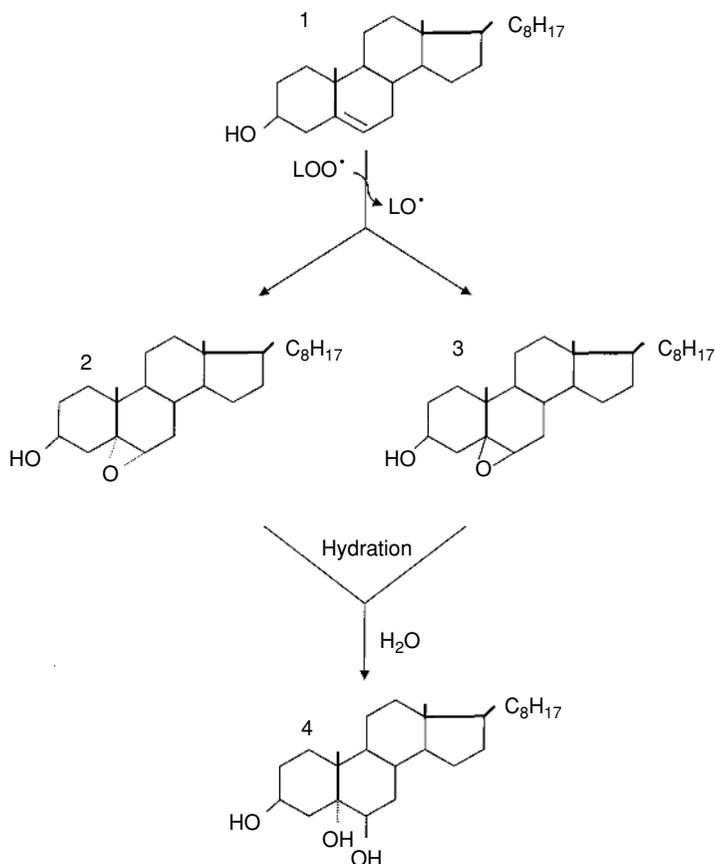
hydroperoxides, transition metal ions, activated oxygen species and radiation (Smith, 1992). The hydroperoxides of PUFAs, formed during lipid oxidation, may be necessary to initiate the oxidation of CHOL. Homolytic scission of hydroperoxides formed in the propagation reaction of lipid peroxidation by the action of transition metal ions ( $\text{Fe}^{2+}$  and  $\text{Cu}^+$ ) could result in the formation of fatty acid peroxy ( $\text{LOO}^\bullet$ ) and alkoxy ( $\text{LO}^\bullet$ ) radicals capable of abstracting the reactive allylic 7-hydrogen atom from CHOL, yielding the carbon-centered radical, 3 $\beta$ -hydroxycholest-5-en-7-yl (7-CHOL $^\bullet$ ) (Figure 18.2), thereby initiating the oxidation process. The 7-CHOL $^\bullet$  radical reacts with  $\text{O}_2$  to yield the 3 $\beta$ -hydroxycholest-5-en-7-peroxy radical (CHOO $^\bullet$ ), stabilized in turn by hydrogen abstraction from an unsaturated fatty acid (LH) or another CHOL molecule to yield epimeric 7-hydroperoxycholesterol (7-OOH), 3 $\beta$ -hydroxycholest-5-ene-7 $\alpha$ -hydroperoxide, 7 $\alpha$ -OOH, and 3 $\beta$ -hydroxycholest-5-ene-7 $\beta$ -hydroperoxide, 7 $\beta$ -OOH (Smith, 1992). The 7-OOH are unstable under conditions involving heat and/or storage and reducing agents. Reducing agents transform the 7-OOH to the corresponding 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterol. In the absence of reducing agents, the reduction of the 7-OOH to the 7-alcohols (7-OH) may be regarded as involving thermal or transition metal ion-catalyzed homolysis of the peroxide bond, yielding cholesterol 7-alkoxy radicals (CHO $^\bullet$ ) (Figure 18.2). The CHO $^\bullet$  radical, in turn, abstracts hydrogens from other molecules to form the major autoxidation OS products, cholest-5-ene-3 $\beta$ ,7 $\alpha$ -diol (7 $\alpha$ -OH) and cholest-5-ene-3 $\beta$ ,7 $\beta$ -diol (7 $\beta$ -OH) (Figure 18.2) (Smith, 1987). Dehydration of the intermediate CHO $^\bullet$  radical results in the formation of 3 $\beta$ -hydroxycholest-5-en-7-one (7-keto). Alternatively, dehydrogenation of 7 $\alpha$ - and 7 $\beta$ -OH leads to the formation of 7-keto derivative, decomposition of which by abstraction of the OH group at position 3, leads to the formation of a conjugated triene, cholesta-3,5-dien-7-one (7-keto-3,5-dien).

Formation of isomeric 5,6-epoxides is mediated by addition of peroxy radicals (arising either from fatty acid hydroperoxides or 7 $\alpha$ - and 7 $\beta$ -OOH) to the 5,6 double bond of CHOL, with subsequent heterolytic cleavage to produce 5,6 $\alpha$ -epoxy-5 $\alpha$ -cholestan-3 $\beta$ -ol ( $\alpha$ -epoxide), and 5,6 $\beta$ -epoxy-5 $\beta$ -cholestan-3 $\beta$ -ol ( $\beta$ -epoxide) (Figure 18.3) (Bortolomeazzi *et al.*, 1994; Chang *et al.*, 1997). Both epoxides are usually detected in oxidized CHOL (Lercker and Rodriguez-Estrade, 2002). Generally, epoxidation of the B-ring with peroxy radicals favors the formation of the  $\beta$ -epoxide rather than the  $\alpha$ -isomer. Hydration of either epoxide, in an acidic environment, generates 5 $\alpha$ -cholestane-3 $\beta$ -5,6  $\beta$ -triol (triol).

CHOL is very stable during heating at 100°C for 24 h, but is unstable at temperatures above 120°C (Osada *et al.*, 1993a). When CHOL is heated at 120°C, 7-keto is the predominant OS formed (Osada *et al.*, 1993a). Heating at 150°C results in the production of OS 7-keto >  $\alpha$ -epoxide



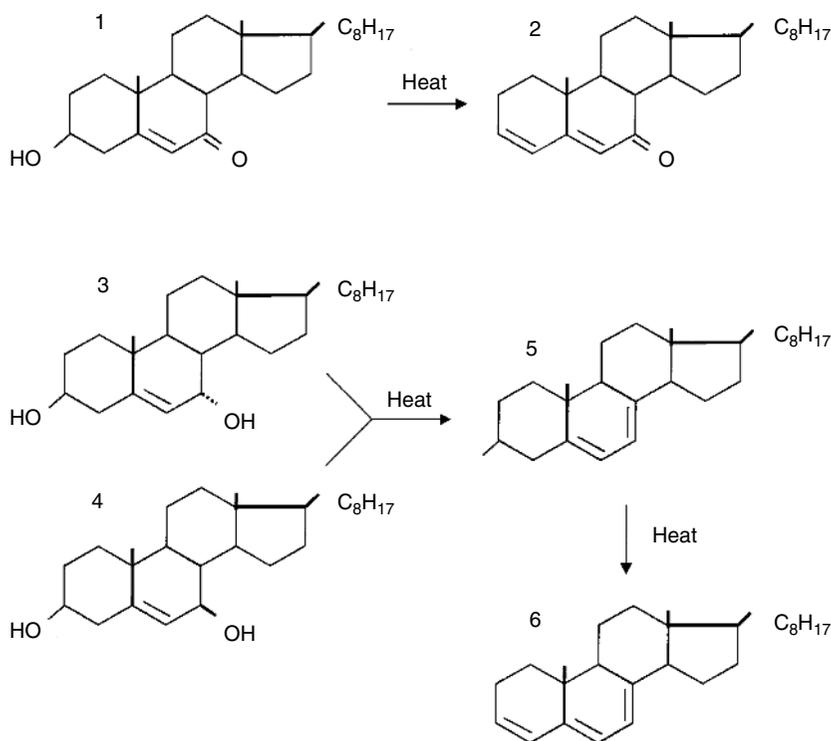
**Figure 18.2.** Cholesterol autoxidation initiated by peroxy (LOO•) or alkoxyl (LO•) radicals arising from peroxidation of polyunsaturated fatty acids (LH). Compounds are as follows: (1) CHOL; (2) 7-CHOL•; (3) CHOO•; (4) 7α-OOH; (5) 7β-OOH; (6) CHO•; (7) 7α-OH; (8) 7β-OH; (9) 7-keto; (10) 7-keto-3,5-dien. For abbreviations, see Table 18.1.



**Figure 18.3.** Scheme for the formation of cholesterol epoxides. Compounds are as follows: (1) CHOL; (2)  $\alpha$ -epoxide; (3)  $\beta$ -epoxide; (4) triol. For abbreviations, see Table 18.1.

$> \beta$ -epoxide  $> 7\alpha$ -OH  $> 7\beta$ -OH. The production of OS at 200°C is low due to the decomposition of CHOL (Osoda *et al.*, 1993a).

When oxidation products such as  $7\alpha$ -OH,  $7\beta$ -OH and 7-keto are heated they can generate other oxygenated compounds (Lercker and Rodriguez-Estrada, 2002). Dehydration of 7-keto, with subsequent abstraction of the OH group at position 3, leads to the formation of a conjugated triene with a keto group, 7-keto-3,5 dien (Figure 18.4). Abstraction of the OH from position 3 is favored by the presence of a double bond between C-5 and C-6 (Lercker and Rodriguez-Estrada, 2002). Elimination of a molecule of water from the OH group at position 7 of  $7\alpha$ -OH and  $7\beta$ -OH, gives rise to

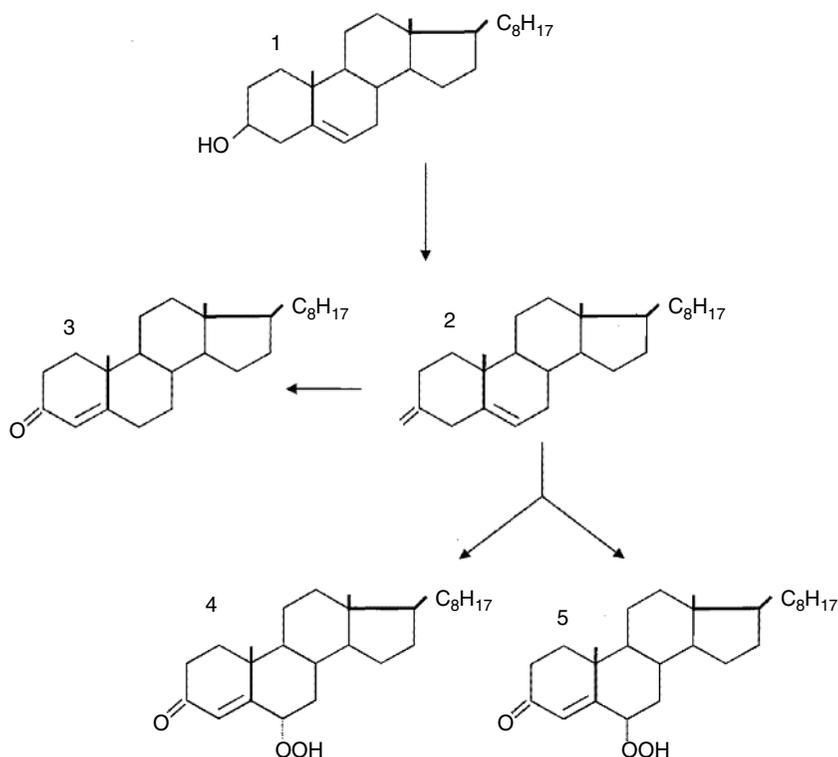


**Figure 18.4.** Effect of heat on some cholesterol oxidation products. Compounds are as follows: (1) 7-keto; (2) 7-keto-3,5-dien; (3); 7 $\alpha$ -OH; (4) 7 $\beta$ -OH; (5) 5,7-dien-3-ol; (6) 3,5,7-triene. For abbreviations, see Table 18.1.

conjugated cholesta-5,7-dien-3 $\beta$ -ol (5,7-dien-3-ol) and subsequently to conjugated cholesta-3,5,7-triene (3,5,7-triene).

Oxidative processes also occur at a very slow rate; they involve oxidation of the 3 $\beta$ -hydroxyl of CHOL and lead to the formation of cholest-5-en-3-one (3-keto-5-en), which rearranges rapidly to cholest-4-en-3-one (3-keto-4-en) with a conjugated double bond structure (Figure 18.5) (Smith, 1987). The 3-keto-5-en can also be oxygenated to the epimeric 6-hydroperoxycholesterols, 3- $\beta$ -hydroxycholest-4-ene-6 $\alpha$ -hydroperoxide (6 $\alpha$ -OOH) and 3- $\beta$ -hydroxycholest-4-ene-6 $\beta$ -hydroperoxide (6 $\beta$ -OOH).

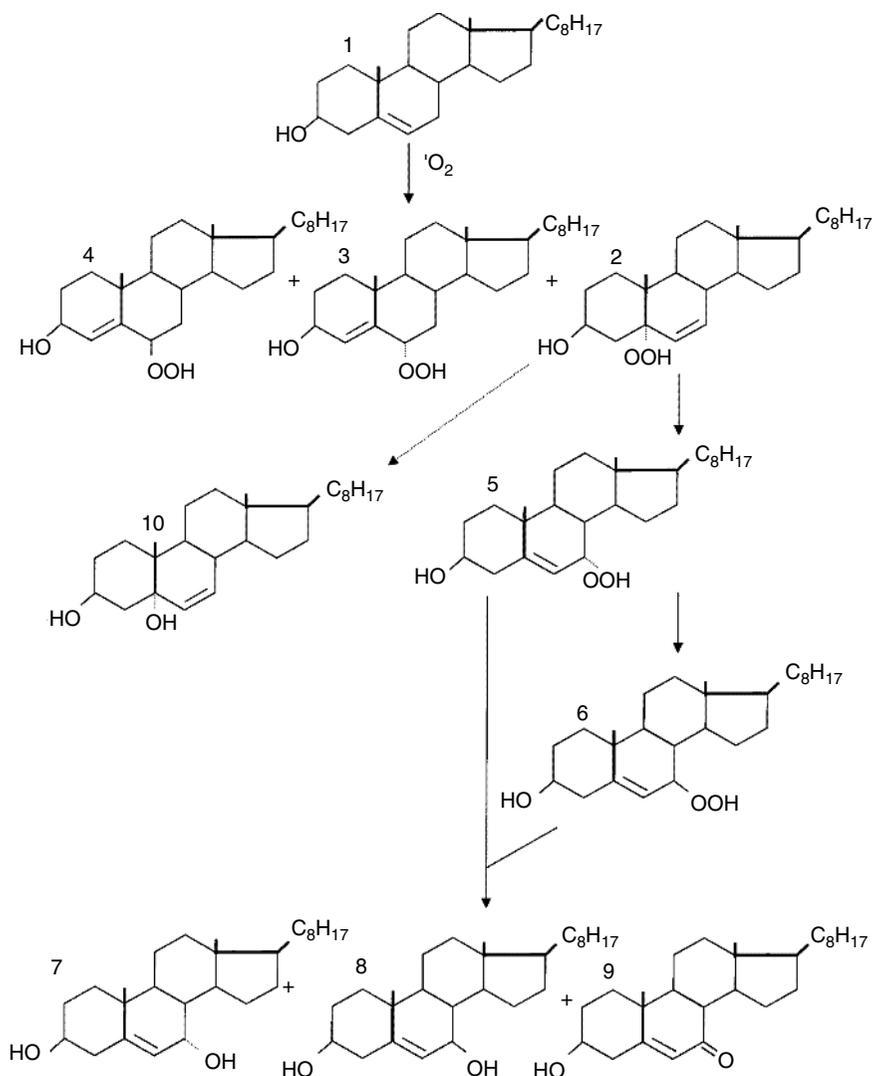
Singlet oxygen, in the excited state ( $^1\text{O}_2$ ), reacts relatively slowly with CHOL *via* a non-radical mechanism; the rate constant for  $^1\text{O}_2$  addition to CHOL is  $\sim 6.7 \times 10^{-4} \text{ mol}^{-1} \text{ s}^{-1}$ , which is about 25% that of methyl arachidonate (Girotti, 2001). Only three hydroperoxides are produced in  $^1\text{O}_2$ -mediated reactions: 3- $\beta$ -hydroxycholest-6-ene-5 $\alpha$ -hydroperoxide



**Figure 18.5.** Formation of some minor cholesterol products. Compounds are as follows: (1) CHOL; (2) 3-keto-5-en; (3) 3-keto-4-en; (4) 6 $\alpha$ -OOH; (5) 6 $\beta$ -OOH. For abbreviations, see Table 18.1.

(5 $\alpha$ -OOH), 6 $\alpha$ - and 6 $\beta$ -OOH (Figure 18.6). The rate of accumulation of 5 $\alpha$ -OOH in photoperoxidizing membranes exceeds that of 6 $\alpha$ - or 6 $\beta$ -OOH by at least five-fold (Smith, 1996; Girotti, 2001). Although  $^1\text{O}_2$  does not produce 7 $\alpha$ -, and 7 $\beta$ -OOH directly, these peroxides could be derived from 5 $\alpha$ -OOH *via* allylic rearrangement and isomerization and, in turn, form 7- $\alpha$ , 7 $\beta$ -OH and 7-keto (Girotti, 2001). Small amounts of decomposition products of 5 $\alpha$ -OOH, such as cholest-6-ene-3 $\beta$ ,5 $\alpha$ -diol (5 $\alpha$ -OH), are also formed *via* the photoxidative mechanism (Smith, 1996).

The effects of riboflavin or fatty acid methyl esters on the photooxidation of CHOL have been investigated (Hu and Chen, 2002; Chien *et al.*, 2003). The data show that the presence of riboflavin or fatty acids methyl esters facilitates the degradation of CHOL and the production of OS. Riboflavin had a more pronounced effect than fatty acid methyl esters. 7-Keto



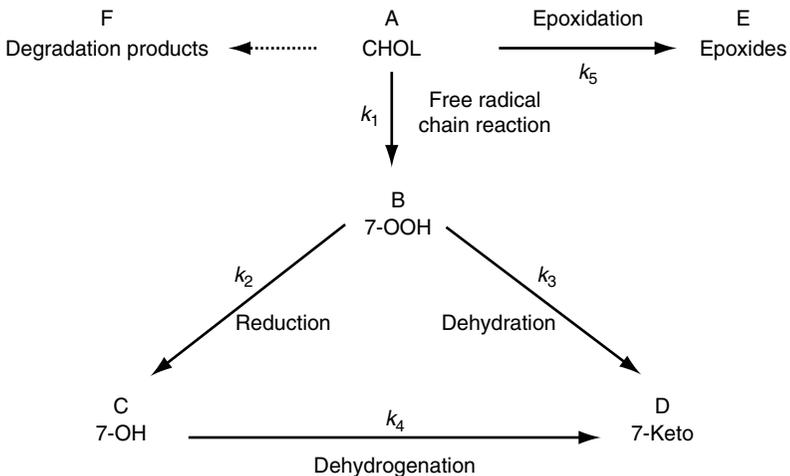
**Figure 18.6.** Main cholesterol oxidation products formed by singlet oxygen. Compounds are as follows: (1) CHOL; (2) 5 $\alpha$ -OOH; (3) 6 $\alpha$ -OOH; (4) 6 $\beta$ -OOH; (5) 7 $\alpha$ -OOH; (6) 7 $\beta$ -OOH; (7) 7 $\alpha$ -OH; (8) 7 $\beta$ -OH; (9) 7-keto; (10) 5 $\alpha$ -OH. For abbreviations, see Table 18.1.

was generated in largest amounts, followed by  $\beta$ -epoxide, 7-keto-3,5 dien,  $\alpha$ -epoxide, 7 $\alpha$ - and 7 $\beta$ -OH. The formation of 7-keto-3,5-dien was probably due to dehydration of 7-keto by energy liberated during illumination (Chien

*et al.*, 2003). The presence of docosahexaenoic ( $C_{22:6}$ ) acid methyl esters favoured the formation of  $\alpha$ - and  $\beta$ -epoxides (Hu and Chen, 2002).

Initiation of oxidation by a water-soluble initiator, 2,2<sup>1</sup>-azobis (2-amidinopropan) dihydrochloride (AAPH), leads to the accumulation of 7-keto as the dominant OS (Nielsen *et al.*, 1996a). The isomeric 7 $\alpha$ - and 7 $\beta$ -OH were also formed in small amounts, and were found to dehydrogenate to 7-keto through a two-step radical reaction. 7 $\alpha$ -OOH, which was formed in higher concentrations than 7 $\beta$ -OOH, was dehydrogenated in the oxidizing system to 7-keto at a rate approximately half of that for 7 $\beta$ -OOH. Nielsen *et al.* (1996a) concluded that 7-keto was not only a product of dehydration of the isomeric forms of 7-OOH, but may also be a product of dehydrogenation of the isomeric forms of 7-OH when exposed to a carbon-centered radical. On the basis of these findings, lipid peroxy radicals formed in foods or in biological systems would appear to be responsible, at least in part, for the generation of 7-keto from the isomeric 7-OOH.

The oxidative mechanisms and pathways for CHOL oxidation are reasonably well documented and are considered to involve a series of free radical chain reactions similar to that for fatty acid oxidation. However, the kinetics of CHOL oxidation has received little attention until recently. Chien *et al.* (1998) defined the major pathways (Figure 18.7) and calculated the rate constants for these reactions (Table 18.2). The reaction can be divided into



**Figure 18.7.** Major pathways of cholesterol oxidation: A, CHOL; B, 7-OOH (7 $\alpha$ - and 7 $\beta$ -OOH); C, 7-OH (7 $\alpha$ - and 7 $\beta$ -OH); D, 7-keto; E, epoxides ( $\alpha$ - and  $\beta$ -epoxides).  $k_1$ - $k_5$ , rate constants of the reactions of cholesterol oxidation. For abbreviations, see Table 18.1.

**Table 18.2.** Rate Constants of the Major Pathways of Cholesterol Oxidation<sup>a</sup>

Equation	Reaction	$k/h^b$
$\text{CHOL} \xrightarrow{k_1} 7\text{-OOH (7}\alpha\text{-and 7}\beta\text{-OOH)}$	free radical chain reaction	$1587 \pm 1$
$7\text{-OOH} \xrightarrow{k_2} 7\text{-OH (7}\alpha\text{-and 7}\beta\text{-OH)}$	Reduction	$781 \pm 107$
$7\text{-OOH} \xrightarrow{k_3} 7\text{-keto}$	Dehydration	$805 \pm 2$
$7\text{-OH} \xrightarrow{k_4} 7\text{-keto}$	Dehydrogenation	$3 \pm 2$
$\text{CHOL} \xrightarrow{k_5} \text{epoxides (}\alpha\text{-and } \beta\text{-)}$	Epoxidation	$1357 \pm 358$

<sup>a</sup> Adapted from Chien *et al.* (1998)<sup>b</sup> Mean  $\pm$  standard deviation

For abbreviations, see Table 18.1.

two major routes: free radical formation at C-7 of CHOL and epoxidation, and one minor route: degradation. Oxidation *via* the C-7 route includes the formation of 7-OOH (7 $\alpha$ - and 7 $\beta$ -OOH) (B) from CHOL (A) through free radical reaction, formation of 7-OH (7 $\alpha$ - and 7 $\beta$ -OH) (C) from 7-OOH; formation of 7-keto (D) from 7-OOH through dehydration and formation of 7-keto (D) through dehydrogenation of 7-OH. The epoxidation route includes the formation of  $\alpha$ - and  $\beta$ -epoxides (E) from cholesterol through 7-OOH, and probably involves the interaction of a hydroperoxyl radical and CHOL (Bortolomeazzi *et al.*, 1994). In addition, CHOL may be degraded to form other products (F). The reactions for the formation of 7-OOH (7 $\alpha$ - and 7 $\beta$ -) and epoxides ( $\alpha$ - and  $\beta$ -epoxides) followed a second-order reaction model, while the formation of 7-OH, 7-keto and the dehydrogenation of 7-OH conformed to a first order reaction model. The data in Table 18.2 show that the value of  $k_1$  is very large, indicating that the formation of 7-OOH increases very rapidly during the initial phase of oxidation, followed by epoxidation, dehydration, reduction and dehydrogenation. The sum of  $k_2$  and  $k_3$  is only slightly lower than  $k_1$ , indicating that 7-OOH is rapidly and quantitatively degraded to form 7-OH and 7-keto. The data also show that the dehydration step (B $\rightarrow$ D) proceeds more rapidly than the reduction step (B $\rightarrow$ C), and that 7-keto is formed, albeit at a slow rate ( $k_3 = 3 \pm 2/h$ ), from 7-OH. This model may help to explain the relatively high concentration of 7-keto compared to other OS products present in many food products. Chien *et al.* (1998) also observed that the epoxides were formed in the greatest amount and increased linearly over the heating period of the kinetic study. They concluded that differences in the heat treatment of cholesterol may greatly affect the formation and degradation rates of 7-OOH.

### 18.2.2. Initiation of Cholesterol Oxidation

Among the potential initiators of CHOL oxidation are pre-formed hydroperoxides, transition metals, peroxy ( $\text{LOO}^\bullet$ ) and alkoxy ( $\text{LO}^\bullet$ ) radicals, hydroxyl radicals ( $\text{HO}^\bullet$ ),  $\text{HOCl}$ ,  $\text{H}_2\text{O}_2$  (Smith, 1996) and nitrogen oxides (Lai *et al.*, 1995). In model systems, cholesterol fatty acyl esters are oxidized by peroxynitrite  $\text{ONOO}^-$  (Van Der Vliet *et al.*, 1994) and copper (Malavasi *et al.*, 1992). The pattern of OS formed generally includes the  $7\alpha\text{-OH}$  and  $7\beta\text{-OH}$  (predominating), 7-keto, 7-keto-3,5-dien,  $\alpha$ - and  $\beta$ -epoxide (predominating), and  $7\alpha\text{-}$  and  $7\beta\text{-OOH}$  (Smith, 1996). The same pattern, without the  $7\alpha\text{-}$  and  $7\beta\text{-OOH}$ , but with the two epoxides ( $\alpha$ -epoxide predominant) arise by  $\text{HO}^\bullet$  attack. Oxidation by  $\text{HOCl}$  produces the two epoxides and cholest-5-ene- $3\beta$ ,  $4\beta$ -diol ( $4\beta\text{-OH}$ ), while several other hydroxyl and keto derivatives were present in smaller amounts (Van den Berg *et al.*, 1993). Zarev *et al.* (1999) observed a markedly lower production of OS (7-keto,  $7\beta\text{-OH}$  and  $\beta$ -epoxide) induced by  $\text{O}_2^\bullet/\text{HO}^\bullet$  free radicals than by copper.

Sterol  $3\beta$ -fatty acyl esters are also susceptible to oxidation, with the formation of three types of oxidized esters: esterified OS, sterol esters of oxidized fatty acyl moieties and OS esters of oxidized fatty acids (Smith, 1996). According to Lercker and Rodriguez-Estrada (2002), cholesteryl esters oxidize more rapidly than CHOL when heated. Oxidation of  $3\beta$ -fatty acyl esters proceeds from initial radical generation at the ester carbonyl group to give esterified OS (Sevilla *et al.*, 1986). Interaction between CHOL and other lipid components, such as triacylglycerol, fatty acid methyl esters and fatty acids, also affect CHOL oxidation (Lercker and Rodriguez-Estrada, 2002). Differences in the susceptibility of CHOL to oxidation in model systems are related to differences in the degree of unsaturation of existing triacylglycerols (Ohshima *et al.*, 1993; Osada *et al.*, 1993b; Li *et al.*, 1994), and a significant linear relationship between the level of OS and peroxide value was also observed (Li *et al.*, 1994). It is interesting to note that oxygen uptake (an index of PUFA oxidation) was not observed when a mixture of triolein and CHOL was incubated (Ohshima *et al.*, 1993). No detectable levels of sterol oxidation products were formed, and the levels of oleic acid also remained unchanged after storage at  $25^\circ\text{C}$  for up to 100 d. However, for mixture of fish liver triacylglycerols and CHOL, a very significant increase in oxygen uptake was observed after 38 d of storage, as well as a continuous increase in OS content with a concurrent decrease in PUFA residues. Lowering the degree of unsaturation of fish oil triacylglycerols was effective in extending the induction phase prior to the propagation phase of CHOL oxidation (Li *et al.*, 1994).

Increasing the degree of unsaturation of PUFA increases the concentration of labile *bis* allylic hydrogen atoms, making it more likely that one of

these hydrogens may be abstracted by  $\text{LOO}^\bullet$  or other radicals (Pryor, 1994; Morrissey *et al.*, 2000). The rate of oxidation of PUFA is proportional to the number of doubly allylic hydrogen atoms in a given PUFA molecule (Pryor, 1994). On a scale where oleate ( $\text{C}_{18:1}$ ) undergoes autooxidation too slowly to measure, linoleate ( $\text{C}_{18:2}$ ), with two allylic hydrogens undergoes oxidation half as fast as does linolenate ( $\text{C}_{18:3}$ ) with four allylic hydrogens. The rate of oxidation of an olefin is expressed as the "Oxidizability Factor" (Pryor, 1994). The oxidizability factor of oleic acid ( $\text{C}_{18:1}$ ) is practically zero and the value for  $\text{C}_{18:2}$ ,  $\text{C}_{18:3}$ ,  $\text{C}_{20:4}$  and  $\text{C}_{22:6}$  is 20, 41, 55 and 102, respectively. Thus, the oxidizability of  $\text{C}_{22:6}$  (10 doubly allylic hydrogen atoms) is five-times greater than that of  $\text{C}_{18:2}$  (two doubly allylic hydrogen atoms). The data of Ohshima *et al.* (1993) and Li *et al.* (1994) clearly indicate that where the oxidizability factor is very low (as for oleic acid), CHOL oxidation is unlikely to occur. On the other hand, where the oxidizability factor is high (as for fish oil, rich in  $\text{C}_{22:5}$ ,  $\text{C}_{22:6}$ ), CHOL oxidation occurs readily. The results strongly suggest that  $\text{LOO}^\bullet$ ,  $\text{LO}^\bullet$  and other radicals from PUFA peroxidation initiate the CHOL oxidation process and promote the propagation stage, probably by abstracting reactive allylic 7-hydrogens from CHOL molecules. The presence of chain-breaking antioxidants, such as vitamin E, inhibits fatty acid and CHOL oxidation in LDL (Halliwell and Gutteridge, 1999), and in muscle-based foods (Monahan *et al.*, 1992; Galvin *et al.*, 1998a).

The oxidation of CHOL induced by ionizing radiation generates a large number of products originating primarily from the reactive allylic 7-hydrogen in CHOL (Sevilla *et al.*, 1986). Both the 25-peroxy and 7-peroxy cholesterol radicals are formed initially at low temperatures, but at higher temperatures, the 25-peroxy radical reacts rapidly, leaving only the 7-peroxy species. Ionizing radiation yields the usual OS, including 7-keto,  $\alpha$ - and  $\beta$ -epoxides and 3-keto-4-en (which originates from the A-ring) and the levels of the isomeric epoxides generally exceed that of 7-keto. Radiation-induced oxidations are also accompanied by rearrangement of the isomeric 5,6-epoxides to 6-keto and of the epimeric diols ( $7\alpha$ - and  $7\beta$ -OH) to 7-keto (Smith, 1996).

### 18.3. Oxysterols in Food Products

During the processing and storage of foods of animal origin, events that result in the formation of fatty acid radicals and hydroperoxides are also likely to lead to the formation of OS. The variables that are important in relation to fatty acid oxidation and OS formation in foods include composition of the food matrix, PUFA content and oxidizability, CHOL level,

processing methods, processing times and temperatures, pH, packaging conditions, composition of the atmosphere, pro- and antioxidants and water activity. Food products that are highly susceptible to CHOL oxidation include milk powders, meat and meat products (including fish), cheese and egg and egg products.

Methodologies employed for the isolation, characterization and quantitation of OS in foods and other biological systems vary considerably in sophistication, with separation and quantitation based on approaches ranging from simple TLC to sophisticated GC, GC-MS, HPLC and NMR methods (Schroepfer, 2000). Extensive reviews have been published on procedures used for sample extraction and purification (Ulberth and Buchgraber, 2002) and determination of OS by GC (Guardiola *et al.*, 2002), HPLC (Rodriguez-Estrada and Caboni, 2002) and by TLC (Lebovics, 2002). Recently, Shan *et al.* (2003) characterized the chromatographic behaviour of a large number of OS on a variety of stationary and mobile phases, and discussed their applications in the isolation and determination of OS in biological systems. Details of methods used for purification and enrichment of OS by various saponification methods and transesterification of lipids were published by Ubhayasekera *et al.* (2004).

Paniangvait *et al.* (1995), in a comprehensive review, noted that research on the occurrence of OS in foods had been based on reliable methods only since the mid 1980s and proposed that there was an urgent need for repeated analyses of OS in foods using standard methods of analysis. Until this is done, many values for OS in foods must be considered approximations. A major deficiency in almost all of the early studies was a lack of serious attention to the problems of artifactual generation of OS from CHOL during processing and analysis of various samples (Rose-Sallin *et al.*, 1995; Schroepfer, 2000). Rose-Sallin *et al.* (1995) presented data on the artifactual generation of OS from CHOL during repeated ( $n = 20$ ) analysis of one sample of milk powder containing 1 mg CHOL per gram. The results indicated that, under the conditions studied, at least 2% of the CHOL in the sample underwent autoxidation during analysis. Specifically, OS levels (ng/g milk powder), formed artifactually, were as follows: 7 $\alpha$ -OH, <10; 7 $\beta$ -OH,  $80 \pm 30$ ; 7-keto,  $1490 \pm 380$ ; triol,  $10 \pm 5$ ; and 25-OH,  $340 \pm 140$  (total OS, 1920).

In an attempt to standardize the analysis of OS, a round-robin test on whole milk powder and skim-milk powder was organised by Appelqvist (1996). Analyses of OS were carried out in 17 laboratories and the differences in the level found led Appelqvist (1996) to conclude that it is still premature to establish "true" values for the level of OS in certain foods. The results of a second round-robin study on egg and milk powders showed that several OS in the samples were not determined or were below the determin-

ation limit of some of the laboratories (Dutta *et al.*, 1999; Dutta and Savage, 2002). The critical control points in the analysis were identified by Dutta and Savage (2002) and include extraction, saponification, enrichment, use of an internal standard, recovery of the OS during work-up steps, response factors, linearity range, limits of detection and quantification. Overall, analysis of OS in foods is a multi-step method and is rather difficult because OS are a minor fraction of unoxidized CHOL (present  $10^3$ - to  $10^6$ - fold in excess) in foods. In addition, CHOL itself is a minor component because it is found associated with bulk lipids such as triacylglycerols, polar lipids and other lipid components. According to Dutta and Savage (2002), small amounts of OS present in foods and the multistep analytical procedures, the lack of authentic standards of defined structure, purity and different approaches taken by different research groups contribute to concerns about the reliability of different published values for the level of OS in the same foods.

A number of review papers have been published recently on the formation and content of OS in eggs and egg products (Galobart and Guardiola, 2002), milk and milk products (Stanton and Devery, 2002), meat and meat products (Kerry *et al.*, 2002), sea-foods and sea-food products (Ohshima, 2002) and in other foods (Evangelisti and Zunin, 2002). Readers are encouraged to explore these reviews for information on the origin and content of OS in food systems.

### 18.3.1. Oxysterols in Dehydrated Systems

Spray-dried, CHOL-containing foods are susceptible to oxidation of the fatty acids and sterols during subsequent storage at ambient temperature for prolonged periods. In general, commercial milk powders contain very low levels of OS (Rose-Sallin *et al.*, 1993; Angulo *et al.*, 1997; McCluskey *et al.*, 1997). The major OS found in whole- and skim-milk powders were the oxidized C-7 OS derivatives,  $7\alpha$ -OH,  $7\beta$ -OH and 7-keto and the total OS level found ranged from 0.26 to 1.9 mg/kg (Rose-Sallin *et al.*, 1995) and from 0.1 to 1.1 mg/kg (Angulo *et al.*, 1997). The latter group observed that storage time at 20°C increased levels of both 7-keto and  $7\beta$ -OH and that the rate of formation of  $7\beta$ -OH was significantly greater than that of  $\beta$ -epoxide (Chan *et al.*, 1993). However, the secondary OS compounds,  $\alpha$ - and  $\beta$ -epoxides and triol, were formed in much higher quantities when the powders were stored at 40°C (Chan *et al.*, 1993) or at 55°C (Angulo *et al.*, 1997). Exclusion of light and oxygen significantly reduced the development of OS in whole milk powders during storage (Chan *et al.*, 1993; McCluskey *et al.*, 1997).

The drying technology used exerts a major influence on triacylglycerol oxidation and on the levels of OS in milk and other powder products, both immediately after drying and upon storage. Nitrogen oxides ( $\text{NO}_x$ ), which

include nitric oxide and nitrous oxide, are produced from air as a result of combustion processes. Morgan and Armstrong (1992) manipulated the levels of NO<sub>x</sub> in the combustion gas of a direct gas-fired heating system and observed that the outlet temperatures and percent NO<sub>x</sub> were the only conditions that affected OS formation in egg yolk powder. Lipid oxidation, including the generation of OS, was also high in spray-dried whole milk powder and tended to correlate with NO<sub>x</sub> levels in the drying air (Chan *et al.*, 1993). Total OS formed in spray-dried whole egg using a direct-heating gas burner (which produced NO<sub>x</sub>) was approximately 2–5 times greater than that in powders processed by an electric heating system (Lai *et al.*, 1995).

### 18.3.2. Oxysterols in High-Fat Products

Nielsen *et al.* (1996b) reported significant accumulation of OS in dairy spreads compared to butter. The concentration of OS was 4 times higher in dairy spreads than in butter after storage at 4°C for 13 weeks, and 7-keto was the dominant oxidation product, at 1.3 and 5.7 μg/g lipid in the stored butter and dairy spread, respectively. This difference in stability is undoubtedly related to the high content of PUFA in dairy spreads.

Home-made ghee, used extensively in traditional Indian cooking, has been estimated to contain up to 12% of total sterol as OS (Jacobson, 1987). However, several studies reported that OS were observed only when the product had been heated at a high temperature and where extensive lipid oxidation had occurred. Epoxides and triol were detected in home-made ghee when the fat was heated at 150°C for 20–25 min, but were absent in commercially processed ghee (Prasad and Subramanian, 1992). Kumar and Singhal (1992) studied the effects of processing conditions on the oxidation of CHOL in cow and buffalo ghee and observed that OS were formed when the samples were clarified at 120°C; the concentration of OS ranged from 0.7 to 0.9% of total CHOL. Intermittent frying increased the level of free fatty acids and the peroxide value (PV) of the ghee, which corresponded to the increase in OS in the samples. All the major sterols (7-keto, 7α-OH, 7β-OH, α-, β-epoxide) increased with frying time, and 20α-OH, 25-OH and triol were detected at low levels (8–16 mg/kg) only after the third frying cycle. Kumar *et al.* (1999) observed that commercial ghee samples contained a small amount of OS (1.32% of total sterol). However, when ghee was heated at 120°C until a PV of ~25 is reacted, OS levels increased to 17.6% of total sterol. Significant increases in the levels of 7-keto, total epoxide, 20α-OH and 3-keto-5-ene were observed.

Butter and ghee are also known to contain a high level of conjugated linoleic acid (CLA) (Sserunjogi *et al.*, 1998), and the content of this compound in ghee can be increased up to fivefold from the base level by increasing the

temperature of clarification from 110 to 120°C (Aneja and Murthi, 1991). Over the past two decades or so, numerous health benefits have been attributed to CLA in humans (Mougiou *et al.*, 2001) and experimental animals (Belury, 2002; see also Chapter 3).

### 18.3.3. Other Factors Involved in Oxysterol Formation

Irradiation is seldom used in the processing of milk and milk products. Nevertheless, it may be appropriate to comment on the effects of  $\gamma$ -irradiation on CHOL oxidation in food systems. In general, irradiation causes a significant increase in OS formation in raw and cooked muscle-based foods when stored under aerobic packaging conditions (Galvin *et al.*, 1998a; Ahn *et al.*, 2001).  $7\alpha$ -OH,  $7\beta$ -OH and 7-keto were detected immediately after irradiation and other secondary products such as epoxides, triol and  $20\alpha$ -OH increased on storage. However, the sensitising effect of irradiation on triacylglycerol and CHOL oxidation is invariably overcome by vacuum packaging.

A strong positive correlation between lipid oxidation and the concentration of OS has been observed in whole milk powder (Chan *et al.*, 1993; McCluskey *et al.*, 1997). Similar relationships have also been observed for muscle-based foods (Monahan *et al.*, 1992; Galvin *et al.*, 1998b). The concentrations of OS and lipid oxidation products (thiobarbituric acid-reactive substances, TBARS) are closely linked to the content of PUFA in model systems (Li *et al.*, 1994) and in meats (Li *et al.*, 1996; Galvin *et al.*, 1998a). The exclusion of oxygen inhibits the initiation of triacylglycerol oxidation, which probably inhibits the subsequent production of OS in food systems during storage for a prolonged period. Elevated  $\alpha$ -tocopherol levels contributed to lower lipid and CHOL oxidation in whole milk powders during storage at an elevated temperature (McCluskey *et al.*, 1997). In addition, antioxidant strategies, which improve the  $\alpha$ -tocopherol status in foods (e.g., muscle-based foods), significantly reduced total OS and the levels of  $7\beta$ -OH, 7-keto and  $\beta$ -epoxide (Monahan *et al.*, 1992; Galvin *et al.*, 2000).

### 18.4. Sources of Oxysterols *In Vivo*

Various OS have been detected in appreciable quantities in human tissue and fluids, including human plasma, atherogenic lipoproteins and atherosclerotic plaque. However, there is still considerable uncertainty regarding the origin of oxidatively-modified sterols (Vine *et al.*, 1998; Brown and Jessup, 1999; Leonarduzzi *et al.*, 2002). OS present *in vivo* may be of exogenous origin (i.e., derived from the diet), or generated endogenously through autoxidation (non-enzymatic oxidation of cholesterol), with a significant

contribution from enzymatic-driven conversion. Some OS of endogenous origin appear to be produced exclusively *via* enzymatic reactions (Bjorkhem *et al.*, 1994).

#### 18.4.1. Absorption of Dietary Oxysterols

Studies on humans (Linseisen and Wolfram, 1998) and animals (Vine *et al.*, 1997, 1998) have demonstrated that dietary OS are efficiently absorbed, mainly in the upper intestinal tract, and then transported in the plasma within chylomicrons (circulating chylomicrons undergo conversion to CHOL-rich chylomicron remnants by the action of endothelial lipoprotein lipase) (Leonarduzzi *et al.*, 2002). After lipolysis, the remnants are cleared rapidly by the liver. Estimates of the extent of OS absorption in rats, rabbits and humans vary greatly (from 93%, Bascoul *et al.*, 1986 to 6%, Vine *et al.*, 1997), which may relate to the dose, model and vehicle used to administer the dose (Brown and Jessup, 1999). There is a suggestion that some OS may be absorbed preferentially and transported by chylomicrons. For example, Emanuel *et al.* (1991) found that 7-keto was the major OS in the plasma of humans fed spray-dried egg powder even though it was not the OS present in the greatest quantity in the eggs. Vine *et al.* (1997) investigated the absorption rate of OS in lymph-cannulated rats and observed that 6% of the OS load was absorbed and incorporated into lymph chylomicrons. The incorporation of OS into lymph chylomicrons differed over time, with 7 $\beta$ -OH having peak absorption at 3 h, followed by 7-keto at 4 h and  $\alpha$ -epoxide at 5 h.  $\beta$ -Epoxide was not detected in chylomicrons. In addition, the OS-treated group had a twofold increase in CHOL and triacylglycerol content compared to rats given purified CHOL. In a follow-up study, Vine *et al.* (1998) who examined the effects of feeding oxidized CHOL (containing 6% OS) or purified CHOL to rabbits over a 2-week period, observed that the group fed oxidized CHOL had 5-times the concentration of  $\alpha$ -epoxide and double the level of 7-keto in triglyceride-rich lipoproteins compared to rabbits fed purified CHOL. The presence of 7-keto in LDL was exclusive to animals fed the oxidized CHOL-rich diet. The study showed that there may be selective absorption into plasma and lymph chylomicrons; in particular,  $\beta$ -epoxide, 3-keto-4-en and 25-OH were not observed in the plasma of the oxidized CHOL-fed rabbits, although these OS were in equal, if not greater, amounts in the feed compared to the  $\alpha$ -epoxides. The concentrations of 7 $\beta$ -OH and 7-keto were similar in both cases, suggesting that a CHOL-supplemented diet may increase endogenous oxidative stress, thereby resulting in increased formation of OS, as suggested by Hodis *et al.* (1991). Differences in the bioavailability of individual OS were also observed by Linseinen and Wolfram (1998).

#### 18.4.2. Oxysterols Formed Endogenously by Nonenzymatic Oxidation

Oxidative processes, similar to those that generate OS in some processed foods, are also likely to occur *in vivo*. Hodis *et al.* (1991) concluded that certain OS (7-keto, 25-OH,  $\alpha$ - and  $\beta$ -epoxide) found at elevated levels in the plasma or aorta of CHOL-fed rabbits were unlikely to be of dietary origin since they could not be detected in the CHOL-containing feed. Hodis *et al.* (1994) studied the OS composition of LDL oxidized *in vivo* and reported very high levels of 7 $\alpha$ - and 7 $\beta$ -OH, 7-keto, 7-keto-3,5-dien,  $\alpha$ - and  $\beta$ -epoxide, triol and 25-OH. It is generally agreed that LDL undergoes oxidation *in vivo* when challenged by a variety of reactive oxygen and nitrogen species and that oxidized LDL is the component central to the initiation and/or progression of atherogenesis at the molecular and cellular level (Steinberg *et al.*, 1989; Ross, 1999). The mechanisms of CHOL oxidation in LDL are still debatable, but are likely to be similar to those that occur in the *in vitro* oxidation of LDL. The typical LDL particle (molecular weight  $\sim 2.5 \times 10^6$  Da) contains  $\sim 1600$  molecules of cholesteryl ester and  $\sim 600$  molecules of free CHOL. About half of the total fatty acids are PUFAs ( $\sim 1300$  molecules), with the primary PUFAs being C<sub>18:2</sub> (86%) and C<sub>20:4</sub> (12%), and a small amount of C<sub>22:6</sub> (2%) (Esterbauer *et al.*, 1992). Vitamin E, mainly as  $\alpha$ -tocopherol, is quantitatively the most important lipophilic antioxidant present in LDL particles. Each LDL particle is protected by 10 molecules of  $\alpha$ -tocopherol (range 3–15 mol), 1 mol  $\gamma$ -tocopherol and small amounts of carotenoids (Esterbauer *et al.*, 1992; Carroll *et al.*, 2000). Vitamin E in the LDL particles acts as a chain-breaking antioxidant and prevents peroxidation of PUFAs and modification of proteins by reactive oxygen species (ROS).

Copper-catalyzed oxidation of LDL is often used to study the effects of oxidized LDL on cells. The oxidative modification of LDL can be divided arbitrarily into three consecutive phases: a lag phase, during which the LDL particles become depleted of  $\alpha$ -tocopherol; a propagation phase, during which the lipid hydroperoxide content of LDL increases rapidly; and a decomposition phase, during which the unstable hydroperoxides are degraded. Chang *et al.* (1997) showed that in the early stages when isolated LDL was exposed to oxidative stress, vitamin E became progressively depleted and the content of conjugated dienes remained low. Small amounts of  $\beta$ -epoxide were formed during the lag phase and the level gradually increased into the propagation phase. Beyond the lag phase, no measurable levels of vitamin E were present, and the levels of conjugated dienes (“footprints” of oxidative stress) increased rapidly, eventually reaching a maximum. As the propagation of lipid peroxidation increased, the accumulation of OS

paralleled the formation of conjugated dienes and OS become major products of the overall oxidation process.  $\alpha$ -Epoxide and  $7\alpha$ -OH accumulated during the latter period of the propagation phase and the levels of 7-keto increased linearly during the entire oxidation period. There is some disagreement concerning the kinetics of OS formation relative to other lipid peroxidation products, as Dzeletovic *et al.* (1995) found that the disappearance of PUFAs and the formation of conjugated dienes preceded the appearance of OS during  $\text{Cu}^{2+}$ -induced oxidation and appreciable OS formation was detected later than conjugated dienes. Results, in general, show that  $\text{C}_{18:2}$  and  $\text{C}_{20:4}$  in LDL particles are consumed in the early stages of incubation with  $\text{Cu}^{2+}$  and the formation of conjugated dienes is more extensive than that of OS. The esterified rather than free CHOL is the more likely target for free radical attack (Dzeletovic *et al.*, 1995; Brown *et al.*, 1996). Thus, free CHOL is most resistant, followed by cholesteryl oleate ( $\text{C}_{18:1}$ ), linoleate ( $\text{C}_{18:2}$ ), arachidonate ( $\text{C}_{20:4}$ ) and cholesteryl docosahexaenoate ( $\text{C}_{22:6}$ ) was the most susceptible to oxidation. During the late final stage of the propagation phase, LOOH and related products with diene conjugated double bonds are substrates for further reactions with metal ions *via* a redox cyclic mechanism to yield  $\text{LOO}^\bullet$  and  $\text{LO}^\bullet$  (Morrissey *et al.*, 1998), that can further drive the OS formation pathways.

Endogenous  $\alpha$ -tocopherol in LDL or supplementation with butylated hydroxytoluene has been shown to prevent (Patel *et al.*, 1996) or delay (Chang *et al.*, 1997) OS formation. This is consistent with previously reported studies where supplementation of diet for humans with vitamin E enhanced the ability of LDL to withstand oxidative stress *in vitro* and only minimal oxidation (as measured by production of conjugated dienes) of LDL occurred while antioxidants were present (Esterbauer *et al.*, 1993). Overall, as pointed out by several research groups (Dzeletovic *et al.*, 1995; Brown *et al.*, 1996, 1997; Patel *et al.*, 1996; Chang *et al.*, 1997), it may be concluded that the oxidation of PUFAs exposed to oxidative stressors *in vitro* or *in vivo* is the likely determining factor in the modification of LDL, and it appears that the oxidation of CHOL in LDL is a secondary and later oxidation event consequent on the attack by  $\text{LOO}^\bullet$  or  $\text{LO}^\bullet$  on the susceptible C-7 allylic position on the B-ring of CHOL. Irrespective of the initiation and propagation methods, the pattern of OS formation was essentially the same in all cases (Patel *et al.*, 1996; Chang *et al.*, 1997). The overall yield of products identified decreased in the order: 7-keto >  $7\beta$ -OH >  $7\alpha$ -OH >  $\beta$ -epoxide >  $\alpha$ -epoxide, except in the case of peroxy-nitrite oxidation, where a higher yield of  $\beta$ -epoxide relative to 7-keto was found (Patel *et al.*, 1996). Side-chain oxidation products of CHOL, including 24-OH, 25-OH and 27-OH, are important components of LDL oxidation.

### 18.4.3. Oxysterols Formed Enzymatically

From a quantitative point of view, the most important OS found *in vivo* are enzymatic products of CHOL metabolism that are involved in the early steps in the conversion of CHOL to bile acids (Schroepfer, 2000). The products of these cytochrome P-450-mediated oxygenations are found in the circulation as dominating sterols, and are involved in the early stages of bile acid formation. The classical, and quantitatively the most important, pathway in the biosynthesis of bile acids in mammalian liver starts with the hydroxylation of CHOL at the  $7\alpha$ -position (Russell, 2000, 2003; Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002; Chiang, 2004). The biosynthetic pathway is under strict metabolic control (hormonal and dietary factors), and multiple mechanisms are involved in the regulation of the rate-limiting hepatic P-450 enzyme, cholesterol  $7\alpha$ -hydroxylase (CYP7A1). In addition to the  $7\alpha$ -hydroxylase pathway, there is an alternative pathway starting with the hydroxylation of cholesterol at C-27 by a mitochondrial cytochrome P-450 (CYP27A1) (Bjorkhem and Diczfalusy, 2002). Because CYP27A1 can oxidize the terminal methyl group not only to a  $\text{CH}_2\text{OH}$  group (27-OH), but also to a carboxylic acid (to form  $3\beta$ -hydroxy-5-cholestenoic acid), this alternative route to bile acid synthesis is called the "acid" pathway. The 27-OH is converted to  $7\alpha$ -OH, 27-dihydroxycholesterol and the carboxylic acid is converted to  $3\beta,7\alpha$ -dihydroxy-5-cholestenoic acid by oxysterol  $7\alpha$ -hydroxylase, mainly in peripheral tissue. CYP27A1 also mediates the conversion of the C-27 steroid side-chain of CHOL to 24- and 25-OH in liver, brain and lung (Bjorkhem, 2002). One of the major OS in the circulation, cholest-5-ene-3  $\beta,4\beta$ -diol ( $4\beta$ -OH), is formed from CHOL in the liver and possibly also in the intestine by the cytochrome P-450 species CYP 3A4. There is some evidence that 7-keto may be produced enzymatically in the liver. However, current thinking suggests that 7-keto present in atherosclerotic plaque is derived only from the diet or produced *in vivo* by free radical attack on CHOL (Lyons and Brown, 1999). The introduction of an oxygen atom into CHOL (formation of OS) drastically reduces its half-life and is a mechanism by which some cells direct excess CHOL to leave the body. OS generated in extrahepatic tissue and organs may be transported to the liver and metabolised to bile acids, that are then excreted into the intestine as water-soluble compounds (Lyons and Brown, 2000). In humans, approximately 400 mg of CHOL per day are converted to bile acids.

OS are able to regulate key enzymes in CHOL turnover at transcriptional and post-transcriptional levels (Wolf, 1999; Bjorkhem, 2000; Tall *et al.*, 2002). CHOL biosynthesis and homeostasis are regulated by two transcriptional factors: steroid regulatory element-binding proteins (SREBP)-1 and -2. These become activated by proteolysis when the CHOL

supply to cells is low and interact with genes that increase enzymes involved in CHOL synthesis [e.g., hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase] (Wolf, 1999). The particular genes are affected by OS at the transcriptional level, HMG-CoA reductase is down-regulated, and CHOL synthesis is reduced (Bjorkhem, 2002; Bjorkhem and Diczfalusy, 2002). When the CHOL level is high, SREBPs become inactive, CHOL biosynthesis stops and the LDL receptor that facilitates the uptake of CHOL into cells is repressed (Wolf, 1999). At the post-transcriptional level, OS may also accelerate the degradation of HMG-CoA reductase because of the presence of high concentrations of  $\text{Ca}^{2+}$ . OS have been identified recently as important physiological activators of LXR and RXR transcription factors that regulate the catabolic degradation of CHOL by activation of the genes controlling cholesterol  $7\alpha$ -hydroxylase, the rate-limiting enzyme in the conversion of CHOL into bile (Wolf, 1999; Tall *et al.*, 2002). LXR and RXR work together to induce a battery of genes that mediate cellular CHOL efflux and transport and CHOL excretion in bile or intestinal lumen.

## 18.5. Biological Effects of Oxysterols

Despite being present at low concentrations, OS are considered to have potent biological effects and have been ascribed a number of important biological functions as metabolic intermediates (Russell, 2000; Schroepfer, 2000), regulators of CHOL homeostasis and other cellular processes (as discussed above), atherogenic agents (Brown and Jessop, 1999; Schroepfer, 2000; Panini and Sinensky, 2001), induction of apoptosis in cells (Panini and Sinensky, 2001) and modulators of cell permeability (Smondryev and Berkowitz, 2001; Meaney *et al.*, 2002). These, and other issues, have been discussed comprehensively by the above authors and consequently only certain issues will be reviewed in this chapter.

### 18.5.1. Effects of Oxysterols on Cell Membranes

When CHOL is incorporated into a lipid bilayer, it preferentially orientates such that  $3\beta$ -OH group interacts with nearby polar head-groups, with the rest of the CHOL molecule oriented roughly perpendicular to the plane of the membrane (Ohvo-Rekila, 2002). This configuration permits the maximum interactions between the non-polar regions of the CHOL molecule and the acyl chains of the bilayer lipids, while minimizing the exposure of the hydroxyl group to a nonpolar environment (Meaney *et al.*, 2002). The introduction of an additional polar moiety (e.g., an OS) into the hydrophobic region of lipid bilayers probably leads to a redistribution of the sterol in conjunction with a local reordering of the acyl chains (Meaney *et al.*, 2002).

The presence of ketocholesterols (Smondirev and Berkowitz, 2001) or side-chain OS (Kauffman *et al.*, 2000) leads to the movement of the OS towards the polar region with the net effect of increasing both the area of the membrane and acyl chain disorder (Smondirev and Berkowitz, 2001). Increasing the area of the membrane is likely to facilitate rapid desorption of an OS from the membrane. For example, the transfer of unesterified 25-OH OS from red blood cells to plasma has been reported to occur about 2000-times faster than that of CHOL (Lange *et al.*, 1995). In addition, the location of the additional oxygen function in OS is of critical importance for its rate of translocation in biological membranes and the rate of its elimination (Meaney *et al.*, 2002). The rate of exchange between erythrocytes and plasma was found to be very high for 27-OH and 24-OH, and extremely low for 4 $\beta$ -OH (structurally similar to CHOL) and for CHOL (Meaney *et al.*, 2002). The rate of transfer of OS from a monolayer to a lipoprotein particle has been shown to follow a clear rank order: 25-OH > 7 $\beta$ -OH > 7-keto (Theunissen *et al.*, 1986). It has been suggested that where the distance between the 3 $\beta$ -OH group and the additional hydroxyl group is large, as in 25-OH or 27-OH, a high rate of OS movement between the inner and outer monolayers of the membrane occurs resulting in high local disordering and high permeability of the membrane (Theunissen *et al.*, 1986; Meaney *et al.*, 2002). On the other hand, when hydroxyl groups are close together in the nucleus (i.e., at positions 3 and 4 or 3 and 7), the disturbing effects on membrane lipids is low and membrane permeability is also low. The observed properties of side-chain oxidized OS may well be linked to their membrane-disturbing effects. Side-chain oxidized OS are cytotoxic and are very potent suppressors of CHOL synthesis (Schroepfer, 2000; Bjorkhem and Diczfalusy, 2002). This effect may be due partly to the down-regulation of HMG-CoA reductase activity by the increased Ca<sup>2+</sup> uptake in human smooth muscle cells (Zhou and Kummerow, 1994). It has been speculated that evolution has favoured metabolic systems that are able to minimize the risk for accumulation of the fast-moving membrane-disturbing OS. The possibility has been discussed that one of the major roles of oxysterol 7 $\alpha$ -hydroxylase is to inactivate 25- and 27-OH by 7 $\alpha$ -hydroxylation, thereby rendering them considerably less toxic.

### 18.5.2. Oxysterols and Apoptosis

OS have been shown to be cytotoxic *in vitro* (Aupeix *et al.*, 1995) and most of the cytotoxicity of freshly isolated LDL is attributable to a minor fraction that has been oxidatively modified and highly enriched in OS (Sevanian *et al.*, 1995). OS are considered to cause injury to endothelial cells, and atheromatous lesions represent “death zones” that contain toxic oxidized lipids and associated OS that cause dysfunction that ultimately

leads to programmed cell death or apoptosis (Li *et al.*, 2001). The exact mechanism of OS-induced apoptosis has yet to be fully elucidated. However, lipid peroxides, OS and aldehydes may increase intracellular steady state levels of ROS, induce modification of cell proteins, and alter various signaling pathways and gene expression (Lizard *et al.*, 2000; Rosenblat and Aviram, 2002; Salvayre *et al.*, 2002; O'Callaghan *et al.*, 2002; Leonarduzzi *et al.*, 2004; Biasi *et al.*, 2004). These events may participate in the toxic effect and trigger an intense, delayed and sustained calcium peak that elicits apoptosis (Salvayre *et al.*, 2002). Disruption of calcium homeostasis is known to play a critical role in toxic cell injury by triggering activation of calcium-dependent degenerative "executioner" caspase enzymes, a family of cysteine-containing proteases, that is known to cause irreversible damage to cellular components and ultimately cell death (Coppola and Ghibelli, 2000; Salvayre *et al.*, 2002).

A number of *in vitro* studies have characterized the potential pro-apoptotic effects of oxidized LDL and the major OS in various cell systems. Oxidized LDL has been shown to induce apoptosis in numerous cell lines, including smooth muscle cells (SMC), endothelial cells, macrophages and lymphoid cells (Auge *et al.*, 2000). Among the OS of pathophysiological interest, 7 $\beta$ -OH and 7-keto induce apoptosis in a variety of vascular cell lines (Lemaire *et al.*, 1998; Miguet *et al.*, 2001; Li *et al.*, 2001) and in a dose-dependent manner (Nishio *et al.*, 1996). Lizard *et al.* (1999) confirmed the effect of 7-keto using cultured SMC obtained from human artery and also observed 7 $\beta$ -OH and 7-keto to be toxic when added to cultures of human umbilical vein endothelial cells (HUVEC). Other OS (25-OH and  $\alpha$ -epoxide) may also induce apoptosis in HUVEC (Lemaire *et al.*, 1998) and apoptosis also occurred after treatment of human monocyte cell lines with either 25-OH or 7 $\beta$ -OH (Aupeix *et al.*, 1995). When 7-keto was added to cells of a macrophage lineage, at a concentration range actually detectable in hypercholesterolemic patients, the pathways of apoptosis were stimulated strongly with cytochrome *c* release, caspase-9 activation and eventually caspase-3 activation (Biasi *et al.*, 2004; Leonarduzzi *et al.*, 2004). However, when identical concentrations of 7-keto were added to the same cells with another OS, namely 7 $\beta$ -OH, or in a mixture with other OS, also detectable in human LDL, the strong pro-apoptotic effect of 7-keto was attenuated markedly. The competition among OS in the mix apparently counteracted the ability of 7-keto given alone to increase greatly the steady levels of ROS in macrophage as well as the up-regulation of the pro-apoptotic factor p21 and the triggering of the mitochondria-dependent pathways of apoptosis (Leonarduzzi *et al.*, 2004). During the very early phases of atherosclerotic lesion formation, various lipid oxidation products (probably including OS) contribute to the up-regulation and expression of inflammatory cytokines

and chemokines in endothelial and smooth muscle cells. In this way, slightly oxidized LDL (containing some OS) could possibly initiate subtle gene modulation within the cell wall without overt vascular cell toxicity and death (Leonarduzzi *et al.*, 2002, 2004; Biasi *et al.*, 2004). In the advanced stages, promotion and progression of atherosclerotic lesions are favoured by reactions that amplify the oxidative modification of LDL through cycles of cell toxicity, repair, proliferation and death (Leonarduzzi *et al.*, 2004). However, the role of OS in this phase has not been defined and there is a need to reevaluate much of the earlier data obtained from *in vitro* studies on cell apoptosis where individual and non-physiological levels of OS were generally used. It is important to recognize also that a composite mix of OS is usually found in oxidized LDL, and is in the presence of a large excess ( $10^3$ – $10^6$ ) of CHOL.

### 18.5.3. Oxysterols and Atherosclerosis

It is generally accepted that the oxidation of LDL is an established hypothesis of atherogenesis and that accumulation of oxidized LDL in the vessel wall is an early event in disease progression (Steinberg *et al.*, 1989; Parthasarathy *et al.*, 1998). Through the oxidation of lipids, oxidized LDL acquire pro-inflammatory properties, that increase smooth muscle cell proliferation and play a critical role in the formation of foam cells (Lusis, 2000; Pryor, 2000; Hayden *et al.*, 2002; Leonarduzzi *et al.*, 2002; Steinberg, 2002). The association between atherosclerosis and OS has recently been extensively reviewed (Brown and Jessup, 1999; Schroepfer, 2000; Garcia-Cruset *et al.*, 2002) and the reader is referred to these reports for detailed descriptions of the role of OS in the atherosclerotic process. OS have been implicated in atherogenesis due to their presence in human atherosclerotic plaque and their potent effect *in vitro* (Brown and Jessup, 1999). *In vitro*, OS activity could be related to atherogenic processes such as prostaglandin synthesis and platelet aggregation, their toxicity to vascular cells, their ability to modify LDL receptor function, their involvement in foam cell formation and advanced phases of atherosclerosis (see above reviews). Several authors have studied the effects of OS added to the diet of animals on the atherosclerotic process. According to Garcia-Cruset *et al.* (2002), the results from these studies seem to be conclusive about the role of OS on the progression of the atherosclerotic lesions. On the other hand, Lyons and Brown (2000) reviewed 16 oxysterol-feeding studies in the literature that used various animal models and concluded that seven were pro-atherogenic, eight anti-atherogenic and one showed no clear effect. Overall, the role of dietary OS in atherosclerosis in animals remains equivocal. Several studies have detected OS in human atherosclerotic plaque (e.g., Carpenter *et al.*, 1995; Brown

*et al.*, 1997), although only relatively few have presented quantitative data (Brown and Jessup, 1999). 27-OH is the major OS in advanced atherosclerotic lesions and was found to be approximately proportional to CHOL levels and increased with increasing severity of atherosclerosis (Carpenter *et al.*, 1995). The 7-oxygenated sterols (7 $\beta$ -OH and 7-keto) appear to be present in foam cells isolated from human atherosclerotic plaque (Mattsson-Hultin *et al.*, 1996) at concentrations at least two orders of magnitude higher than those of plasma (Brown and Jessup, 1999). Recently, Garcia-Cruset *et al.* (2002) reported that traces of OS, such as  $\alpha$ - and  $\beta$ -epoxides, 7 $\alpha$ - and 7 $\beta$ -OH, 7-keto and 27-OH, were present in normal human arteries, whereas total OS (standardized to CHOL) was much higher in fatty streaks and highest in advanced atherosclerotic plaque. Plasma OS have also been shown to be higher in smokers than in non-smokers and long-term vitamin E supplementation has been shown to be effective in reducing the plasma level of 7 $\beta$ -OH (Porkkala-Saratoha *et al.*, 2000).

Based on observational studies, Jacobson (1987) proposed that the markedly higher incidence of heart disease in Indians living in London compared with non-Indians in the same city may be related to their high consumption of ghee, which was reported to contain very high concentrations of OS (12.3% of total sterols). Recent studies in Britain have identified a greater degree of insulin resistance in children of South Asian ancestry compared with white children (Whincup *et al.*, 2002). Gupta *et al.* (2004) also observed that in Asian Indians there are increasing trends in the prevalence of metabolic syndrome, also known as cardiovascular dysmetabolic syndrome. Metabolic syndrome is a constellation of abnormalities characterized by central obesity, high triacylglycerol, hypertension or high normal blood pressure, low levels of high-density lipoprotein (HDL) CHOL and diabetes. Subjects with metabolic syndrome are at increased risk of developing diabetes mellitus and cardiovascular disease as well as increased mortality from other causes. Indian Asians living in the UK have approximately 50% higher rate of mortality from coronary heart disease than the native Caucasian population, which, essentially, can be attributed to metabolic syndrome (Brady *et al.*, 2004). Thus, the present knowledge points to the prevalence of various components of metabolic syndrome rather than dietary OS as the critical contributors to atherogenesis in Asian Indians living in the UK.

There is still no clear evidence that OS contribute directly to atherogenesis in humans. According to Brown and Jessup (1999), further studies are necessary to determine definitely the role of OS in atherosclerosis, and Bjorkhem and Diczfalusy (2002) concluded that the normal dietary intake of OS is probably of little or no importance in the development of atherosclerosis. It is possible that OS associated with modified LDL may be a marker or "footprint" of late events or may be an additional factor

accompanying, rather than causing, inflammation and fatty streak lesions in the arterial wall.

## 18.6. Conclusions

CHOL and its derivatives undergo autoxidation *via* a free radical mechanism and the first free radicals formed appear to be located at position 7. The process of CHOL oxidation is probably initiated in foods and other biological systems *via* free radicals generated from PUFA oxidation that abstract a hydrogen atom to form the initial CHOL radicals. Increasing the degree of unsaturation of coexisting triacylglycerols increases the susceptibility of CHOL to oxidation and reduces the induction phase. On the other hand, chain-breaking antioxidants, such as vitamin E, inhibit the oxidation of both PUFA and CHOL and innovative processing technologies and packaging and storage systems that help to maintain a favourable prooxidant-antioxidant balance are likely to prevent the formation of OS in foods. The available evidence indicates that the amount of OS ingested from conventionally processed foods is very low, and is unlikely to play a significant part in the development of atherosclerosis and other biological changes. OS are formed in LDL oxidized *in vitro*, and are also present in atherosclerotic plaque; however, there is no direct evidence yet that OS formed *in vivo* contribute to the development of atherosclerosis in humans. Differences have been reported on the degree of cytotoxicity from one OS to another and their ability to induce apoptosis in a variety of vascular cell types. These conclusions have been based mainly on data obtained from *in vitro* experiments with pure OS at levels of dubious pathophysiological relevance. The recent reports that competition among OS, present at normal physiological levels, diminish ROS induction and the triggering of apoptosis will no doubt provide a stimulus for further studies. Finally, OS are formed *in vivo* by enzymic mechanisms, and it has been reported that they may be important regulators of CHOL homeostasis. Understanding the mechanisms whereby OS regulate macrophage CHOL efflux and intestinal excretion may have potential for the treatment and/or prevention of atherosclerosis.

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# High Performance Liquid Chromatographic and Gas Chromatographic Methods for Lipid Analysis

K.N. Kilcawley

## 19.1. Introduction

Milk fat contains a number of different lipids, but is predominately made up of triacylglycerols (TAG) (98%). The remaining lipids are diacylglycerols (DAG), monoacylglycerols (MAG), phospholipids, free fatty acids (FFA) and sterols. Milk fat contains over 250 different fatty acids, but 15 of these make up approximately 95% of the total (Banks, 1991); the most important are shown in Table 19.1. The unique aspect of bovine, ovine and caprine milk fat, in comparison to vegetable oils, is the presence of high levels of short-chain volatile FFAs (SCFFA), which have a major impact on the flavor/aroma of dairy products. Most cheeses are produced from either bovine, ovine or caprine milk and the differences of their FFA profile are responsible for the characteristic flavor of cheeses produced from such milks (Ha and Lindsay, 1991).

Milk fat composition and esterase/lipase activity are also very important in the dairy industry in terms of flavor, functionality and nutrition. Therefore, it is necessary to have accurate methods to enable quantification of the key milk fat components and lipolytic activity. Methods specifically relating to the quantification of esterase and lipase activity are described in detail in Chapter 11. However, methods incorporating chromatography for the determination of lipolytic activity are described briefly in this chapter

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

**Table 19.1.** Principal free fatty acids in milk fat

Fatty Acid	Notation	% (w/w)
Butyric (Butanoic acid)	C <sub>2:0</sub>	4.2
Caproic (Hexanoic acid)	C <sub>4:0</sub>	2.9
Caprylic (Octanoic acid)	C <sub>6:0</sub>	1.5
Capric (Decanoic acid)	C <sub>8:0</sub>	3.7
Lauric (Dodecanoic acid)	C <sub>12:0</sub>	4.4
Myristic (Tetradecanoic acid)	C <sub>14:0</sub>	12.3
Palmitic (Hexadecanoic acid)	C <sub>16:0</sub>	31.3
Stearic (Octadecanoic acid)	C <sub>18:0</sub>	3.5
Oleic ( <i>cis</i> -9-Octadecenoic acid)	C <sub>18:1</sub>	19.5
Linoleic ( <i>cis</i> -9, <i>cis</i> -12-Octadecadienoic acid)	C <sub>18:2</sub>	1.8
Linolenic ( <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatrienoic acid)	C <sub>18:3</sub>	1.2

together with a detailed account of chromatographic methods used to quantify individual FFAs, MAGs, DAGs and TAGs.

## 19.2. Quantification of FFAs

### 19.2.1. Gas Chromatography

The most widely used method for quantifying FFAs is gas chromatography (GC), which has attained widespread favor due to its versatility, high sensitivity and relatively low cost. GC complexed with a flame ionization detector is used routinely to quantify FFAs, either directly or derivatized as fatty acid methyl esters (FAME). GC with mass spectroscopic detection has become the favored technique for quantification of volatile compounds derived from lipids (esters, lactones, ketones, alcohols and acids).

Over the last 20 years, the selectivity and separation efficiency of columns has increased markedly with the advent of capillary wall coated open tubular (WCOT) columns, which have in many cases made the original packed GC columns redundant. Capillary columns are very narrow (0.1–0.3 mm internal diameter) and typically 25–50 m long. They consist of fused silica on a flexible polymeric coat with a thin internal bonded liquid phase. The choice of internal phase depends on the nature of material to be resolved and the narrow diameter and length give optimum partitioning of the sample constituents (Christie, 2003a). The thickness of the film (liquid phase) has a marked impact on retention time, the thicker the film, the longer the retention time, but the greater the capacity of the column. Film thickness can vary from 0.1 to 5.0  $\mu\text{m}$ ; the general rule is that low boiling point compounds

require a thin film, while volatile compounds require a thick film. As the fatty acids are in the gaseous phase (usually helium or hydrogen), they travel down the column with the carrier gas and diffuse into the liquid phase to varying degrees according to their equilibrium constant and are thus separated.

Modern GCs have an automatic injector which greatly reduces premature evaporation of the sample, which occurred with manual injection. There are a number of different types of injection systems, such as split injection, splitless injection, on-column injection and programmed-temperature injection. Split injectors are used widely to extend the life of WCOT columns, which are prone to overloading. In this system, after injection and vaporization, the sample vapors are mixed with the carrier gas. The gas line is split between the column and the split-line; only a small volume of gas (sample) enters the column, as most is vented off to the atmosphere. The amount of sample loaded onto the column and the volume passing through the split-line is controlled by the split ratio (Rood, 1999a). This system is designed to introduce the sample to the column rapidly as the injector has a very high gas flow rate. A major disadvantage of this technique is that it may discriminate between FFAs due to their wide range of boiling points.

In splitless injection, the sample is not split as most of it enters the column and the remainder is vented to the atmosphere. It is used mainly for the analysis of trace compounds. In this system, the sample is injected and vaporized with a solvent and mixed with the carrier gas in the injection chamber. The solvent typically has a high boiling point relative to the column temperature and will condense when it enters the column, forming a narrow band containing the sample. After a pre-set time, the split line is opened which maintains the narrow band of sample at the top of the column (Rood, 1999a; Christie, 2003a). Most injectors operate in either split or splitless mode. With on-column injection, a liquid sample is deposited directly onto the column without vaporization. Discrimination problems are avoided since the amount of each compound entering the column depends on its concentration, not volatility. The primary purpose of an on-column injector is to guide the syringe needle into the column (Rood, 1999a). In this procedure, care must be taken to not overload the column; therefore, sample dilution must be gauged correctly prior to injection (Christie, 2003a). Temperature-programmable injectors are widely available and offer the analyst an enhanced opportunity to resolve, and thus quantify, components with similar properties. In this system, the injector can be heated very rapidly during a GC run. This is useful for samples containing compounds with a wide range of volatilities. The system is typically used in conjunction with on-column injection where components move from the injector to the column once they reach their boiling point as the injection temperature is increased (Rood, 1999a; Christie, 2003a).

The most commonly used detector for FFA analysis is the flame ionization detector (FID) due to its versatility, sensitivity and stability. Organic compounds exiting the column enter a hydrogen-oxygen flame. Combustion of the compounds in the flame creates ionic species, which are attracted to a charged collector, creating a current. This current is measured as a signal and differences are registered as peaks (Rood, 1999b). An example of a modern GC-FID system is shown in Figure 19.1.

### 19.2.2. High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is the most widely used chromatographic technique. Its popularity is due to high separation power, excellent selectivity and the high diversity of analytes that can be quantified (Cserháti and Forgács, 1999). The main difference between HPLC and GC is the use of a liquid mobile phase and different methods of detection. Quantification of components depends on the type of technique used, column, mobile phase, temperature, flow rate, and most importantly, type of detector. Spectrometric detectors in the ultra violet (UV)/visible range are the most widely used. The most useful types for lipid analysis are those that offer a range of variable wavelengths, the best being photodiode array detectors. Spectrophotometric detectors can analyze only a few lipid components directly, but are widely used in conjunction with suitable derivatization methods (Christie, 2003a). Refractive index and evaporative



**Figure 19.1.** A Varian 3800 gas chromatograph coupled to a flame ionization detector and a Varian CP-8400 automatic injector operated by Varian Star Workstation Software Version 5.

light-scattering detectors are also used, but are not as common due to their high cost.

Reverse-phase (RP) HPLC has been used widely for FFA analysis. The stationary phase is almost always the octadecylsilyl (ODS) type. The mobile phase is typically acetonitrile or methanol in water and detection is by UV between 205 and 210 nm. FFAs are separated on the basis of both chain length and degree of unsaturation (Christie, 1997). An example of a modern HPLC system is shown in Figure 19.2.

### 19.2.3. Isolation of FFA

Quantification of FFAs in dairy products, especially in cheese, is particularly important due to the impact of some FFAs on flavor. However, FFAs act as precursors of a wide range of flavor compounds (e.g., methyl ketones, lactones, esters and aldehydes), (Singh *et al.*, 2003). The extent of lipolysis in cheese varies widely between varieties (Table 19.2). Typically, those cheeses with more than  $\sim 3000$  mg/kg have a characteristic lipolytic aroma/flavor and lipolysis plays an important role in their ripening. A major difficulty in quantifying FFAs in cheese is the distribution of FFAs of different chain length within the cheese matrix. SCFFA ( $C_{4:0}$ – $C_{8:0}$ ) partition mainly into the aqueous phase, whereas medium ( $C_{10:0}$ – $C_{14:0}$ ) and longer



**Figure 19.2.** A Waters 2695 high performance liquid chromatograph with a Waters 2487 dual absorbance detector operated by Waters Empower Software.

**Table 19.2.** Average free fatty acid content (mg/kg) of a number of important cheese varieties

Cheese Variety	FFA (mg/kg)	Reference
Blue (US)	32,230	Woo <i>et al.</i> , 1984b
Brick	2,150	Woo <i>et al.</i> , 1984b
Brie	2,678	Woo <i>et al.</i> , 1984b
Cabrales	57,266	de la Feunte <i>et al.</i> , 1993
Caciocavallo Silano	15,171	Corsetti <i>et al.</i> , 2001
Caerphilly	1,253	McNeill and Connolly, 1989
Camembert	681	Woo <i>et al.</i> , 1984b
Cheddar	1,028	Woo <i>et al.</i> , 1984b
Cheshire	1,265	McNeill and Connolly, 1989
Colby	550	Woo <i>et al.</i> , 1984b
Edam	356	Woo <i>et al.</i> , 1984b
Emmental	2,206	McNeill and Connolly, 1989
Gorgonzola	31,600	Contarini and Toppino, 1995
Gruyere	1,481	Woo <i>et al.</i> , 1984b
Idiazabal	5,577	de la Feunte <i>et al.</i> , 1993
Jarlsberg	3,538	Kilcawley (unpublished)
Limburger	4,187	Woo <i>et al.</i> , 1984b
Mahon	8,743	de la Feunte <i>et al.</i> , 1993
Majorero	20,794	de la Feunte <i>et al.</i> , 1993
Manchego	32,404	Poveda <i>et al.</i> , 1999
Monterey Jack	736	Woo <i>et al.</i> , 1984b
Mozzarella	583	Kilcawley (unpublished)
Munster	6,260	de Leon-Gonzalez <i>et al.</i> , 2000
Parmesan	4,993	de la Feunte <i>et al.</i> , 1993
Pecorino Romano	6,311	Bütikofer, 1996
Picante	17,161	Freitas and Malcata, 1998
Port Salut	700	Woo <i>et al.</i> , 1984b
Provolone	2,118	Woo and Lindsay, 1984
Roncal	8,178	de la Feunte <i>et al.</i> , 1993
Roqueforti	32,453	Woo <i>et al.</i> , 1984b
White Pickled Cheese	603	Akin <i>et al.</i> , 2003

(C<sub>16:0</sub>–C<sub>18:3</sub>) chain FFAs partition with the fat (IDF, 1991). Therefore, the choice of method to isolate or separate FFAs from the cheese matrix is very important if all FFAs are to be determined accurately. Typically, separated FFAs are then quantified by either GC or HPLC. Over the years, many different chromatographic methods have been used to quantify FFAs but these are relatively few in comparison to the methods employed to isolate FFAs from cheese.

### 19.2.3.1. Steam Distillation

Kosikowski (1977) described a distillation method for extracting volatile FFAs from cheese. However, individual FFAs were not quantified as the extract was titrated to a specific end point, with the amount of alkali used relating to the level of volatile acids present. Steam distillation was used successfully by Horwood and Lloyd (1980) to isolate FFAs from cheese. Formic acid was used to form FFAs from the salts obtained after distillation of the acids from cheese into alkali. This method was also used by Parliament *et al.* (1982) who extracted SCFFAs from an acidified aqueous suspension of cheese. Contarini *et al.* (1988) evaluated steam distillation for the extraction of volatile FFAs from Grana cheese and obtained very good recoveries. Kilcawley *et al.* (2001) also used steam distillation to isolate C<sub>2:0</sub>, C<sub>3:0</sub> and C<sub>4:0</sub> from enzyme-modified cheese.

### 19.2.3.2. Solvent Extraction and Solid-Liquid Partitioning

The methods for isolation of FFAs fall generally into two categories; solvent extraction or solid-liquid partitioning, but in many cases combinations of both have been used. Due to differences in the solubility of fatty acids in solvents, care must be taken to select those which offer the best opportunity for extracting the whole range of FFAs. Solubility increases as the chain length of the fatty acids in lipids decreases, or as the chain length of the solvent alcohol increases. The most commonly used solvents for extraction are diethyl ether and hexane. Their wide use is due predominantly to their low toxicity and the high solubility of SCFFAs therein. Spangelo *et al.* (1986) devised a method for isolating FFAs involving their extraction from milk using acetonitrile in the presence of H<sub>2</sub>SO<sub>4</sub> (reduced pH aids extraction of the FFAs) and anhydrous Na<sub>2</sub>SO<sub>4</sub> which binds moisture also aids the extraction of FFA. The mixture was filtered and dried. FFAs were methylated using a strong anion-exchange resin prior to analysis by GC. Contarini *et al.* (1988) extracted FFAs from Taleggio cheese using ethyl ether-petroleum ether (40–60%, v/v) after the addition of anhydrous Na<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>SO<sub>4</sub>. The salts of the FFAs were obtained by titration with ethanolic NaOH, dried and converted to FFAs prior to GC analysis.

### 19.2.3.3. Supercritical fluid extraction (SFE)

In this technique, highly compressed CO<sub>2</sub> above its critical pressure is used to extract FFAs, followed by chromatography. The density of a supercritical fluid resembles that of a liquid, but its viscosity is similar to that of a gas (Christie, 2003a). Its main advantage is the ability to adjust solvent power by regulating pressure and temperature. CO<sub>2</sub> is used widely due to

its low toxicity. Tuomala and Kallio (1996) used this technique to separate volatile compounds from Swiss cheese using on-line GC-FID and mass spectrometry. SCFFAs were extracted, but some minor volatile components co-eluted with some of the FFAs, interfering with resolution.

#### 19.2.3.4. Solid supports

A number of different solid supports have been used to isolate FFAs from dairy products. Early methods used alkaline or acid silicic-based columns (Nieuwenhof and Hup, 1971; Gray, 1975). However, Stark *et al.* (1976) reported that such columns may hydrolyse TAGs. Woo and Lindsay (1982) used a similar approach to isolate FFAs from a range of dairy products using a modified silicic acid-KOH column, which included a preliminary step to remove lactic acid that interfered with the subsequent chromatography of SCFFAs. FFAs were eluted from the column using a solution of 2% formic acid in diethyl ether and concentrated by rotary evaporation prior to GC analysis. This method was used to quantify FFAs in several cheese varieties (Woo and Lindsay, 1984; Woo *et al.*, 1984a). The method was modified by Woo *et al.* (1984b) who used an ethylene glycol pre-column to remove lactic acid. However, this pre-column was reported to be sensitive to water and therefore  $\text{MgSO}_4$  instead of  $\text{Na}_2\text{SO}_4$  should be used with samples with a high moisture content to increase the life of the column.

Other investigators have used an anion exchange resin as solid support (Bills *et al.*, 1963; Deeth *et al.*, 1983; Needs *et al.*, 1983; McNeill *et al.*, 1986; McNeill and Connolly, 1989; de Jong and Badings, 1990; Ha and Lindsay, 1990).

Bills *et al.* (1963) used pre-treated Amberlite resin dispersed in hexane to isolate FFAs from milk. Fat was removed from the resin using hexane, absolute ethanol and methanol and the FFAs were esterified prior to analysis by GC. Needs *et al.* (1983) extracted lipids from milk by using ether and the FFAs were isolated using a strong basic anion exchange resin (Amberlyst 26, BDH Ltd, Poole Dorset, UK). The FFAs were methylated and resolved by GC. McNeill *et al.* (1986) also used Amberlyst resin to isolate FFAs in conjunction with silicic acid to remove phospholipids. Extracted FFAs were then analyzed by GC. This method was used by McNeill and Connolly (1989) to quantify FFAs in a number of semi-hard cheeses.

Deeth *et al.* (1983) isolated FFAs from milk and dairy products by first extracting the FFAs using diethyl ether, or mixtures of hexane-diethyl ether under acidic conditions in the presence of  $\text{Na}_2\text{SO}_4$ . These extracts were then passed through a glass column containing deactivated alumina to remove neutral TAGs. The alumina with the adsorbed FFAs was dried. Di-isopropyl ether containing 6% formic acid was added, mixed and centrifuged. The

supernatant, containing the FFAs, was analysed by GC. de Jong and Badings (1990) also used alumina as adsorbent, but modified the procedure of Deeth *et al.* (1983) by using 3% formic acid in diethyl ether. They reported a higher recovery and better repeatability than Deeth *et al.* (1983). FFAs ( $< C_{12:0}$ ) and total branched chain FFAs in cheese and milk fat were analyzed by Ha and Lindsay (1990), who also used neutral alumina to isolate FFAs after the fat was extracted in diethyl ether:hexane (1:1, v/v). FFAs were eluted from the alumina using formic acid in di-isopropyl ether. SCFFAs were separated from long-chain FFAs by simultaneous distillation-extraction. Butyl esters of the FFAs were prepared and quantified by GC.

de Jong and Badings (1990) also evaluated a solid phase aminopropyl column conditioned in heptane to isolate FFAs from milk prior to quantification by GC. Milk fat, extracted with ether:heptane (1:1, v/v) was added to the aminopropyl column and the neutral lipids removed by chloroform:2-propanol (2:1, v/v). The FFAs were eluted from the column using diethyl ether containing 2% formic acid. The authors compared this method to the alumina method and found considerably higher recovery of all FFAs. FFAs were quantified directly by capillary GC. Further work by de Jong *et al.* (1994) highlighted the need to use an excess of adsorbent aminopropyl, as some dairy samples contain a high level of lactic acid, which has an affinity for aminopropyl columns, reducing binding capacity for FFAs. They also demonstrated that interference in the resolution of FFAs by GC caused by lactic acid is reduced as only a very low level of lactic acid elutes with the FFAs. A collaborative study using numerous extraction procedures for FFAs highlighted the robustness of aminopropyl columns (Bütikofer, 1996). This study recommended the use of hexane:2-propanol (3:2, v/v) instead of chloroform:2-propanol (2:1, v/v) due to the toxicity of chloroform. It was also found that most commercial aminopropyl cartridges are washed with acetic acid ( $C_{2:0}$ ) prior to packaging and therefore the level of  $C_{2:0}$  varies between batches, making it difficult to quantify  $C_{2:0}$  by this procedure.

A novel method for the determination of SCFFAs in cheese was reported by Innocente *et al.* (2000). Grated cheese was mixed with water and crotonic acid. The mixture was centrifuged and the aqueous phase acidified to pH 3 or 4 to aid the extraction of FFA salts. The FFAs were then extracted in diethyl ether and determined by GC.

#### 19.2.4. GC Analysis

Gas chromatographic analysis of FFAs isolated from dairy products has been performed by a number of different techniques. Examples of

methods used to quantify FFAs by GC involve injecting the FFAs directly (Nieuwenhof and Hup, 1971; Contarini *et al.*, 1988; de Jong and Badings, 1990; Innocente *et al.*, 2000), or indirectly in the form of potassium salts dissolved in formic acid (Horwood and Lloyd, 1980; Gray, 1975), or as volatile butyl esters (Parodi, 1970; Ha and Lindsay, 1990) or as methyl esters (Martín-Hernández *et al.*, 1988; McNeill and Connolly, 1989).

Innocente *et al.* (2000) quantified volatile FFAs directly in a diethyl ether extract, with crotonic acid as an internal standard, on a Nukol fused-silica wide-bore GC column. This method is rapid and reproducible for the quantification of acids from C<sub>2:0</sub> to C<sub>7:0</sub>. The method of Gray (1975) involved titration of isolated FFAs with methanolic KOH and concentrating by drying. The FFAs were extracted in a mixture of acetone and water, then analyzed using a packed GC column containing diethylene glycol succinate. Parodi (1970) prepared butyl esters of FFAs from milk fat. Fat was extracted by hexane, mixed with butanolic KOH and saturated with NaCl. This solution was centrifuged and the top hexane layer, containing the butyl esters, then separated on a packed GC column. Ha and Lindsay (1990) prepared butyl esters by drying FFAs previously isolated from cheese in diethyl ether under nitrogen. BF<sub>3</sub>/butanol was added to the dried mixture which was then boiled for 10 min, after which 10 ml of methanol:water (15:100, v/v) and 10 ml of pentane were added and the solution mixed and centrifuged. The resulting butyl esters of the FFAs were concentrated prior to analysis on a capillary GC column. Bills *et al.* (1963) prepared methyl esters from FFAs previously extracted from milk fat by adding ethylene chloride, then saturating with NaCl. The ethylene chloride layer, which contained the methyl esters, was concentrated prior to GC analysis in a packed column.

However, the methods most widely used for quantification of FFAs in dairy products are adaptations of the methods of Martín-Hernández *et al.* (1988) and de Jong and Badings (1990). Martín-Hernández *et al.* (1988) developed a rapid method to quantify FFAs in cheese. Lipids were extracted from an acidified cheese paste with diethyl ether and tetramethylammonium hydroxide (TMAH). An upper phase contains transesterified TAGs, while the lower phase contains TMA soaps of FFAs, which were subsequently converted to methyl esters in the GC injector. This procedure was developed originally with a packed column, but excess TMAH can pyrolyze to trimethylamine and interfere with chromatographic resolution. Interference due to trimethylamine was eliminated by using a capillary WCOT column (typically chemically bonded polyethylene glycol) and a temperature programmable injector with an initial temperature of 300°C (Juárez *et al.*, 1992). Chavarri *et al.* (1997) found that the FFA fraction should be separated from the TAG fraction prior to derivatization with TMAH as transesterified

TAGs can transfer to the lower phase and be converted to methyl esters, giving false high results.

In the method described by de Jong and Badings (1990), FFAs are isolated using an aminopropyl column; the isolated extract is injected directly onto a capillary GC column using cold on-column injection. The stationary phase of the capillary column is typically any nitroterephthalic acid-modified, chemically bonded polyethylene glycol capillary column; however, it is necessary to use a column with good film thickness ( $>0.5 \mu\text{m}$ ) to prolong performance of the column. Direct on-column injection without cooling is possible but reduces the longevity of the column considerably. Chavarri *et al.* (1997) found that this method gave 90–100% recovery of FFAs regardless of their chain length or the ratio of FFAs to TAGs. A major advantage of this method is that it is readily amenable to automation and that the use of on-column injection eliminates discrimination between FFAs of different volatility.

The methods described above have been used principally to quantify FFAs in cheese, but can be used for other milk products with some slight modifications. All the above methods use internal standards (typically FFAs which are not present in milk fat), and the recovery of all FFAs is based on the recovery of these internal standards. It is best to use both volatile and non-volatile FFAs as internal standards. Currently, the International Standard for the extraction of lipids and lipo-soluble compounds from milk and milk products is ISO 14156 (ISO, 2001) and involves solvent extraction. Determination of the fatty acid composition of milk fat involves the preparation of fatty acid methyl esters (FAME) by transesterification (ISO, 2002a), followed by quantification by GC (ISO, 2002b).

### 19.2.5. HPLC Analysis

One of the first HPLC methods to resolve the major FFAs in milk fat used RP-HPLC (Reed *et al.*, 1984). In this method, FFAs in milk are converted to *p*-bromophenacyl esters *via* a crown ether-catalyzed reaction. Two chromatographic runs were required to quantify all FFAs due to problems of co-elution of some medium- and long-chain FFAs. However, Elliott *et al.* (1989) accomplished complete separation of all FFAs in one run using this method with a gradient of acetonitrile in water at a column temperature of 10°C. Further development of the method was undertaken by Garcia *et al.* (1990), who used a water/methanol/acetonitrile gradient to achieve faster separation of all FFAs. This method is one of the most widely used for quantifying FFAs by HPLC. FFAs, without esterification, can also be separated by HPLC using an ODS stationary phase column and acetic or phosphoric acid in the mobile phase to suppress ions (Christie, 2003a). This

method appears to have been applied mainly to vegetable fats to detect adulteration (Hein and Isengard, 1997).

Sanches-Silva *et al.* (2004) compared RP-HPLC and GC-FID methods to quantify FFAs in potato crisps. Sample preparation for the RP-HPLC method involved saponification of the FFAs with 0.5M NaOH in ethanol-water (94:4, v/v). The ethanol dissolves the sodium salts of FAs, which are then separated from other salts and water-soluble impurities using 0.6 M HCl. The sodium salts of the FAs are converted to free acids and then analyzed on an ODS2 column using a gradient of acetonitrile and water. The GC-FID technique involved the production of FAME followed by separation on a polyethyleneglycol capillary GC column. The authors found very little difference between the methods for the quantification of the long-chain FFAs, C<sub>18:1</sub>, C<sub>18:2</sub> and C<sub>18:3</sub>. The RP-HPLC method may be applicable to the wide range of FFAs in dairy products.

A useful fluorogenic derivatization method for long-chain FFAs in milk was described by Lu *et al.* (2002). The FFAs were converted to fluorescent naphthoxyethyl derivatives and separated by isocratic HPLC and monitored using a fluorometric detector at an excitation wavelength of 235 nm and an emission wavelength of 350 nm. Other fluorometric detection methods appear to be used mainly to quantify long-chain FFAs in vegetable oils. Kotani *et al.* (2002) described an HPLC method for quantifying FFAs using electrochemical detection. The basis of the method involves the voltametric reduction of quinone, after it has been associated with FFA. The method does not require derivatization and separation is achieved using an ODS column. The method has been only applied to the isolation of long-chain FFAs, but appears to be reproducible and sensitive. Kilcawley *et al.* (2001) quantified C<sub>2:0</sub>, C<sub>3:0</sub> and C<sub>4:0</sub> acids in cheese and in enzyme-modified cheese using a monosaccharide hydrogen ionic column (ion-partitioning) with an isocratic mobile phase of 0.1 N H<sub>2</sub>SO<sub>4</sub> with detection at 220 nm, after the SCFFAs had been isolated by steam distillation.

### 19.2.6. Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) has attracted much interest recently, due its potential anti-cancer, anti-atherogenic and anti-inflammatory therapeutic properties (Weiss *et al.*, 2004; see Chapter 3). CLA occurs as several positional and geometric isomers of C<sub>18:2</sub> with a conjugated double bond. The *cis*-9, *trans*-11 CLA isomer occurs most often naturally. Commercial sources of CLA also contain the *trans*-10 and *cis*-12 isomer, which is also biologically active. Since various isomers of CLA differ in biological activity, methods of analysis for the various CLA isomers have become very

important. It is necessary to be able to separate and quantify each geometrical and positional isomer and to avoid isomerization during any derivatization steps. CLA is commercially available as a free acid in large amounts and is therefore relatively easy to quantify. However, naturally-occurring CLA is present in its esterified form at very low concentrations which makes it much more difficult to quantify (Christie, 2003b).

Capillary GC is widely used to quantify CLA. Best results are attained using base-catalyzed rather than acid-catalyzed transesterification due to geometrical isomerization, which increases the relative proportions of all *trans* isomers. Depending upon the nature of the sample, capillary polyethylene glycol columns will achieve sufficient separation of the main CLA isomers, but minor isomers may co-elute. Best separation of CLA isomers is achieved using high polarity polyethylene glycol capillary columns with a stabilized cyanopropyl or cyanosilicone phase (Christie, 2003b). Silver-ion HPLC is also very useful for separating and quantifying positional and geometrical isomers of CLA. UV quantification of methyl esters separated by silver-ion HPLC is used routinely. Silver-ion thin-layer chromatography (TLC) is often required for the enrichment of CLA isomers prior to GC analysis. GC-MS is used widely to identify specific isomers (Christie, 2003b).

### 19.3. Lipid-Derived Volatile Aroma and Flavor Compounds

Lipid-derived volatile compounds play an important role in the flavor of foods. These compounds contribute to the characteristic notes of many dairy flavors, but are also responsible for many off-flavors. Parliament and McGorin (2000) reviewed those volatile compounds important in milk, cream, butter, cultured creams and cheese. The pathways involved in the degradation of milk fat have also been reviewed by McSweeney and Sousa (2000) and compounds include FFAs, methyl ketones, lactones, esters, aldehydes, primary and secondary alcohols, hydroxyacids, hydroperoxides and ketoacids.

#### 19.3.1. Gas Chromatography Mass Spectrometry

Virtually all volatile aromatic and flavorsome lipid-derived compounds are analyzed using gas chromatography-mass spectrometry (GC-MS). The components of interest are isolated initially, concentrated, then injected onto a suitable capillary column and detected using a mass spectrometer.

The principle of GC-MS involves the bombardment of organic molecules of interest in the vapor phase with electrons to form positively charged ions, which fragment in a number of different ways to give smaller ionized entities. These ions are propelled through magnetic or electrostatic fields and



**Figure 19.3.** A Varian 3400 gas chromatograph with a Tekmar 3000 purge-and-trap concentrator and a Varian Saturn 2000 mass spectrometer detector operated by Varian Saturn GC/MS Workstation Software Version 5.41 k.

separated according to their mass to charge ratio (Christie, 2003a). Mass spectrometry is used widely as a mass-selective GC detector and is unequalled as an identification tool (Reineccius, 2002). An example of a modern GC-MS system is shown in Figure 19.3.

### **19.3.2. Isolation and Concentration of Volatile Lipid-Derived Components**

Numerous techniques have been developed to isolate and concentrate aroma compounds derived from lipids or other components, from other constituents than dairy foods. The most widely used methods are based on volatility and/or solubility.

Methods based on volatility include static and dynamic headspace analysis, high vacuum and steam distillation and direct injection (Reineccius, 2002). Static headspace involves extracting the vapor of a food under vacuum and directly injecting it onto a GC capillary column, followed by mass spectrometry; however, the method lacks sensitivity. Dynamic headspace methods usually involve purge-and-trap. These methods use an inert gas (helium or nitrogen) to purge a sample, releasing volatile constituents, which are then trapped on sorbants (Tenax, charcoal, activated carbon) or cryogenically (liquid nitrogen). The approach can be problematic as the choice of trapping material can discriminate between compounds and the use of cryogenics

causes sample dilution due to the entrapment of water (Reineccius, 2002). Problems can also occur due to the complexity of the equipment and contamination of traps.

Distillation techniques, such as steam distillation, are typically carried out in a rotary evaporator after the sample has been solubilized in an organic solvent. The distillate is injected directly onto a suitable GC column. This method is used widely due to its simplicity and because components with high boiling points are recovered easily. High-vacuum distillation is used widely to isolate volatile components from solvent extracts. This procedure often requires an extraction step to remove water.

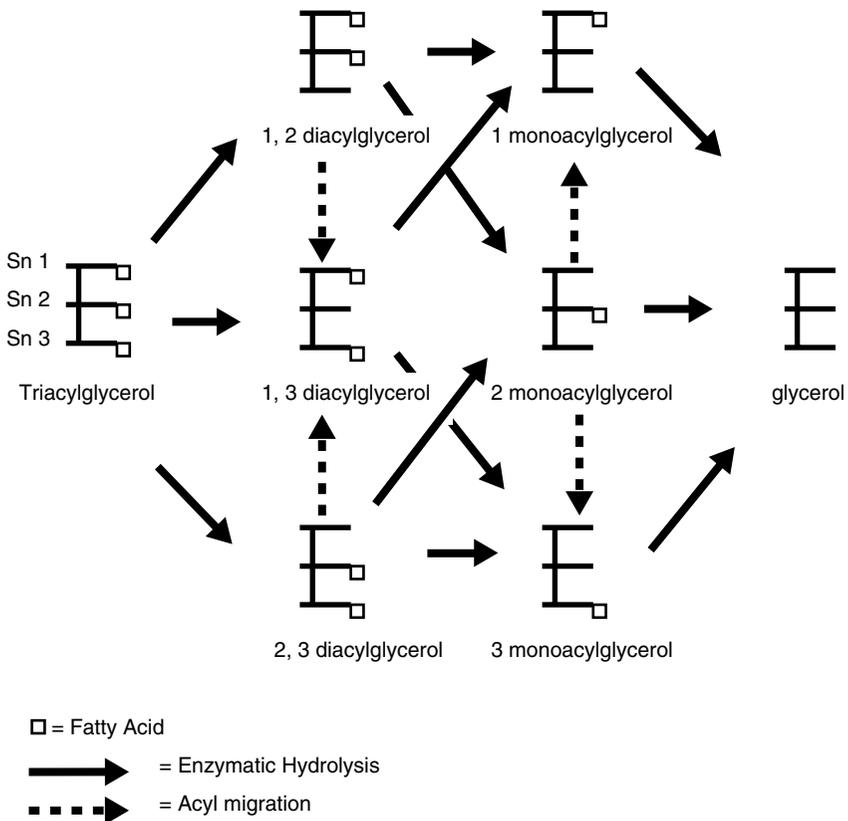
Direct solvent extraction techniques are widely used and range from very simple procedures where solvent and sample are mixed and dried to the use of supercritical CO<sub>2</sub> and pressure chambers. The efficiency of the technique is influenced greatly by the choice of solvent.

Solid phase micro-extraction (SPME) allows isolation and concentration of volatile components rapidly and easily without the use of a solvent. These techniques are independent of the form of the matrix; liquids, solids and gases can be sampled quite readily. SPME is an equilibrium technique and accurate quantification requires that the extraction conditions be controlled carefully. Each chemical component will behave differently depending on its polarity, volatility, organic/water partition coefficient, volume of the sample and headspace, speed of agitation, pH of the solution and temperature of the sample (Harmon, 2002). The techniques involve the use of an inert fiber coated with an absorbant, which govern its properties. Volatile components are adsorbed onto a suitable SPME fiber (which are usually discriminative for a range of volatile components), desorbed in the injection chamber and separated by a suitable GC column. To use this method effectively, it is important to be familiar with the factors that influence recovery of the volatiles (Reineccius, 2002).

Odor-active components in cheese flavor, many of which are derived from milk lipids, can be detected using GC-olfactometry (GC-O). GC-O is defined as a collection of techniques that combine olfactometry, or the use of the human nose, as a detector to assess odor activity in a defined air stream post-separation using a GC (Friedrich and Acree, 1988). The data generated by GC-O are evaluated primarily by aroma extract dilution analysis or Charm analysis. Both involve evaluating the odor activity of individual compounds by sniffing the GC outlet of a series of dilutions of the original aroma extract and therefore both methods are based on the odor detection threshold of compounds. The key odourants in dairy products and in various types of cheese have been reviewed by Friedrich and Acree (1988) and Curioni and Bosset (2002).

### 19.4. Tri-, Di- and Mono-Acylglycerols

TAGs consist of a glycerol backbone, with the three hydroxyl groups esterified to a fatty acid. They are by far the most abundant lipids in animal and vegetable fats. Most lipolytic enzymes cleave TAGs at the *sn*-1 and *sn*-3 positions, with relatively few enzymes showing a preference for the *sn*-2 position. Removal of one fatty acid produces a DAG, removal of two produces a MAG and removal of three FAs produces a glycerol molecule. 2,3- and 1,2-DAGs and 2-MAGs are inherently unstable and undergo acyl migration to 1,3-DAGs and 1-MAGs, respectively (Kilara, 1985) (see Figure 19.4).



**Figure 19.4.** Enzymatic degradation of triacylglycerol to diacylglycerols monoacylglycerols and glycerol.

Due to the great diversity of their molecular species, it is difficult to separate all species by one method alone. The unstable nature of some DAGs and MAGs increases this difficulty. TAGs, DAGs and MAGs can be separated by TLC on silica gel using various mixtures of hexane, diethyl ether, acetic or formic acid as the solvent (Christie, 2003a). TAGs, DAGs and MAGs are separated based on their overall polarity and TLC is widely used to isolate and purify TAG fractions. Another useful method to separate TAGs, DAGs and MAGs is silver ion TLC where separation is achieved according to the number of double bonds in the acyl residues. RP-TLC has been used widely to separate TAGs according to their polarity. A combination of silver ion-TLC and RP-TLC has been used to elucidate the structure of TAGs (Nikolova-Damyanova, 1999). High temperature gas chromatography has also been used widely to separate TAGs according to the sum of the molecular weights of their constituent fatty acids. This type of analysis requires columns with coating materials stable up to 380°C; various non-polar and polar columns have been used successfully to analyze TAGs of various vegetable and butter oils. Fused silica open tubular columns appear to give the best separation of TAG according to the distribution of the double bonds on the fatty acyl moieties and by the molecular mass of each TAG.

Chromatographic and spectrophotometric methods used for the analysis of TAGs have been reviewed by Andrikopoulos (2002). Details are given of HPLC (normal, RP, silver-ion, size exclusion/gel permeation), GC, supercritical fluid chromatography, mass spectrometry and TLC methods. Laakso (2002) has also reviewed mass spectrometric methods used to characterize TAGs. Most of the above methods have been used to characterize TAGs, DAGs and MAGs in vegetable oils and therefore are concerned mainly with TAGs containing predominantly long-chain FFAs. Some methods dealing specifically with TAGs, DAGs and MAGs in milk fat have been reported. Contarini and Toppino (1995) studied TAGs, DAGs and MAGs in Gorgonzola cheese. Fat was extracted in hexane and diethyl ether, followed by TLC. Chromatograms were developed using 2,7-dichlorofluorescein in ethanol. The bands corresponding to TAGs, DAGs or MAGs were recovered and extracted with chloroform and diethyl ether. MAGs and DAGs were separated as trimethyl silane derivatives using GC and TAGs were chromatographed directly. García-Ayuso and Luque De Castro (1999) compared chromatographic and non-chromatographic techniques for the quantification of TAGs and FFAs in dairy products. Nájera *et al.* (1999) achieved good resolution for many TAGs in cheese by a RP-HPLC method with a light scattering detector.

## 19.5. Chromatographic Methods for the Analysis of Lipolytic Activity

Fatty acids released by lipases can be determined quantitatively by TLC, GC and HPLC. TLC methods are used in conjunction with densitometric methods or autoradiographic methods using radiolabelled TAG. Many GC and HPLC methods that have been outlined earlier are widely used to isolate and quantify FFAs in lipolytic assays. Additionally a method using *p*-nitrophenyllaurate as a substrate was described by Maurich *et al.* (1991) who quantified activity by the release of *p*-nitrophenol. Veeraragavan (1990) used a RP-HPLC method with triolein as the substrate. Triolein was emulsified in buffer with the aid of a surface active agent and the lipase added under controlled conditions. Lipolytic activity was measured by the release of oleic acid and quantified by absorbance at 208 nm.

Patel *et al.* (1996) produced trimethylsilyl derivatives of FFAs, MAGs, DAGs and TAGs in butterfat hydrolyzed by different lipases. GC was used to quantify these compounds; the method enabled FFAs of acyl carbon numbers from 4 to 18, MAGs with acyl carbon numbers of 12–18, DAGs with acyl carbon numbers of 16–36 and TAGs with acyl carbon numbers of 30–46 to be identified.

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# Spectroscopic Techniques (NMR, Infrared and Fluorescence) for the Determination of Lipid Composition and Structure in Dairy Products

É. Dufour

Spectroscopic techniques have been used increasingly in the agricultural and food industries. In recent years, it has become increasingly clear that the application of spectroscopic methods to food analysis can solve important problems in the processing and distribution of food and food products. Indeed, traditional analytical methods for major food components are relatively expensive, time-consuming, require highly-skilled operators and are not easily adapted to online monitoring. Moreover, many chemical methods of analysis are not effective enough to cover the growing demands of industry. To meet this need, a great number of noninvasive and nondestructive instrumental techniques, such as nuclear magnetic resonance (NMR), infrared (IR) and fluorescence spectroscopy have been developed to determine product composition and properties. These new analytical techniques are relatively low-cost and can be applied in both fundamental research and in the factory as on-line sensors for monitoring dairy products.

## 20.1. Spectroscopic Techniques Used to Study Fats in Dairy Products

### 20.1.1. Infrared Spectroscopy

Infrared radiation refers to energy in the region of the electromagnetic spectrum at wavelengths longer than those of visible light, but shorter than

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

radio waves. The application of infrared spectroscopy in animal nutrition, agricultural and food sciences have increased considerably in the last decade (Givens *et al.*, 1997; Givens and Deaville, 1999).

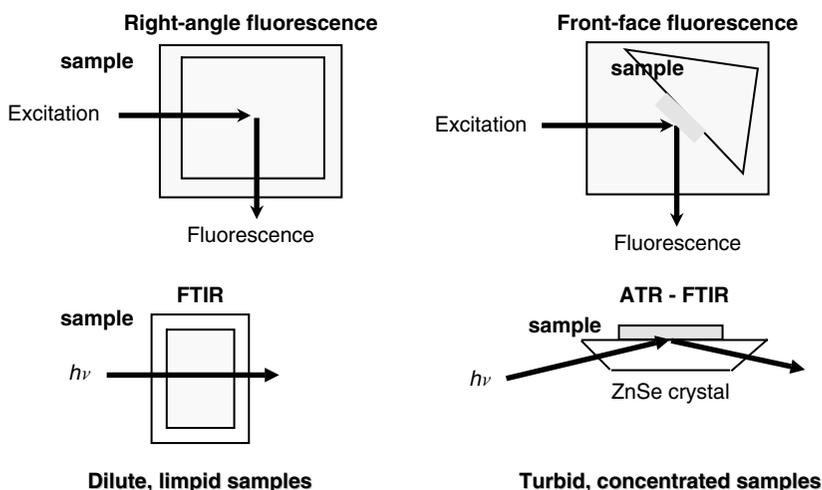
Near infrared (NIR) spectroscopy is used widely for the determination of organic constituents in feeds, foods, pharmaceutical products and related materials. The technique is advantageous for many applications because it can provide rapid, nondestructive and multi-parametric measurements. The technique is also suitable for at-line and on/in-line process control, and is based on the absorption of electromagnetic radiation at wavelengths in the range 800–2500 nm. NIR spectra of foods correspond mainly to overtones and combinations of vibrational modes involving C–H, N–H and O–H chemical bonds. One of the strengths of NIR technology is that it allows several constituents to be measured simultaneously (Rodríguez-Otero *et al.*, 1997). The superposition of many different overtone and combination bands in the NIR region causes a very low structural selectivity for NIR spectra compared to the mid-infrared (MIR) radiation where many fundamentals can usually be observed in discrete positions.

MIR represents the spectrum of absorption of all chemical bonds between 4000 and 400  $\text{cm}^{-1}$ . Most of the absorption bands in the mid-infrared region, but not in the near-infrared region, have been identified and attributed to chemical groups. The absorbance of C–O ( $\sim 1175 \text{ cm}^{-1}$ ) and C=O ( $\sim 1750 \text{ cm}^{-1}$ ) of the ester bonds of triacylglycerols and the acyl chain C–H ( $3000\text{--}2800 \text{ cm}^{-1}$ ) are commonly used to determine fat. The infrared bands appearing in the  $3000\text{--}2800 \text{ cm}^{-1}$  region are particularly useful because they are sensitive to the content, the conformation and the packing of the triglycerides (Casal and Mantsch, 1984; Dufour *et al.*, 2000). For example, the phase transition of phospholipids (sol-to-gel state transition) can be followed by mid infrared spectroscopy; increasing temperature results in a shift of the bands associated with C–H ( $\sim 2850, 2880, 2935$  and  $2960 \text{ cm}^{-1}$ ) and carbonyl stretching mode of phospholipids.

Due to the high absorbency in the MIR region and to comply with the Beer–Lambert law, the path-length of the cuvette must be about 10  $\mu\text{m}$ . The development of the attenuated total reflectance (ATR) device allows the sampling problems encountered when collecting spectra from opaque and viscous samples to be overcome (Figure 20.1).

### 20.1.2. Fluorescence spectroscopy

Absorption of light by a fluorescent molecule causes the excitation of an electron moving from a ground state to an excited state (Lakowicz, 1983). After the electron has been excited, it relaxes rapidly from the higher vibrational states to the lowest vibrational state of the excited electronic state after which, the excited state may decay to the ground state by the



**Figure 20.1.** Light paths in fluorescence and infrared spectroscopies for the investigation of dilute solutions and food products.

emission of a photon (fluorescence). Due to energy losses, the emitted fluorescence photon is always less energetic than the absorbed photon (Lakowicz, 1983).

Fluorescence spectroscopy offers several inherent advantages for the characterization of molecular interactions and reactions. Firstly, it is 100–1000 times more sensitive than other spectrophotometric techniques. Secondly, fluorescent compounds are extremely sensitive to their environment. For example, vitamin A that is buried in the hydrophobic interior of a fat globule has fluorescent properties different from molecules that are in an aqueous solution. This environmental sensitivity enables characterization of viscosity changes such as those attributable to the thermal modifications of triglyceride structure, as well as the interactions of vitamin A with proteins. Third, most fluorescence methods are relatively rapid (less than 1 s with a Charge Coupled Device detector). One particularly advantageous property of fluorescence is that one can actually see it since it involves the emission of photons. The technique is suitable for at-line and on/in-line process control.

If absorbance is less than 0.1, the intensity of the emitted light is proportional to the concentration of fluorophore and excitation and emission spectra are accurately recorded by a classical right-angle fluorescence device. When the absorbance of the sample exceeds 0.1, both emission and excitation spectra are reduced and excitation spectra are distorted. To avoid

these problems, the method of front-face fluorescence spectroscopy can be used to record the spectra of samples with high absorbance ( $OD > 2$ ) such as milk and cheese (Figure 20.1).

Fluorescence probes or fluorophores represent the most important area of fluorescence spectroscopy. Fluorophores can be divided broadly into two main classes: intrinsic and extrinsic. Intrinsic fluorophores, such as vitamin A, are those which occur naturally. Extrinsic fluorophores are added to the sample to provide fluorescence when none exists or to change the spectral properties of the sample. Very apolar probes such as 9,10-diphenylanthracene and diphenylhexatriene (DPH) have been tested by Herbert (1999) to label the lipids in dairy products. These probes are specific for lipids, but are susceptible to severe photo-bleaching upon exposure to the UV light of a laser. BODIPY<sup>®</sup> 665/676, which offers an unusual combination of apolar structure and long-wavelength fluorescence with the excitation wavelength set at 640 nm, has been used in confocal microscopy studies to label fat globules in coagula (Herbert, 1999).

### 20.1.3. Nuclear Magnetic Resonance

NMR is a versatile spectroscopic technique for studying opaque heterogeneous samples, which has already been proven to have a number of useful applications in dairy research (Duce *et al.*, 1995). The technique is also suitable for at-line and on/in-line process control. NMR is based on the magnetic properties of the nucleus of certain atoms, such as the nucleus of the hydrogen atom,  $^1H$ , the nucleus of carbon-13,  $^{13}C$ , and the nucleus of phosphorus-31,  $^{31}P$ . It is convenient to divide the parameters obtained from NMR spectra into static and dynamic parameters.

Static parameters are obtained from the observed resonance frequencies, the precise value of which is determined by molecular properties. For isotropic systems, the two most important parameters determining the resonance frequency are chemical shift and scalar spin-spin coupling. Considering a spectrum, the area under a given peak is proportional to the number of spins contributing to the signal, allowing the determination of concentration. In addition, dynamic processes at the molecular level influence the nuclear spin system by rendering the spin Hamiltonian time-dependent. This means that, depending on the relation between the characteristic times of the molecular motions and the strength of the modulated interactions, one can identify different regimes. For slow motions, the system is in the solid state. For fast motion systems, the spin relaxation is characterized by a limited number of time constants,  $T_1$  and  $T_2$ . The NMR parameters,  $T_1$ , the spin lattice or longitudinal relaxation time constant, and  $T_2$ , the spin-spin or transverse relaxation time constant of protons are related with the relative

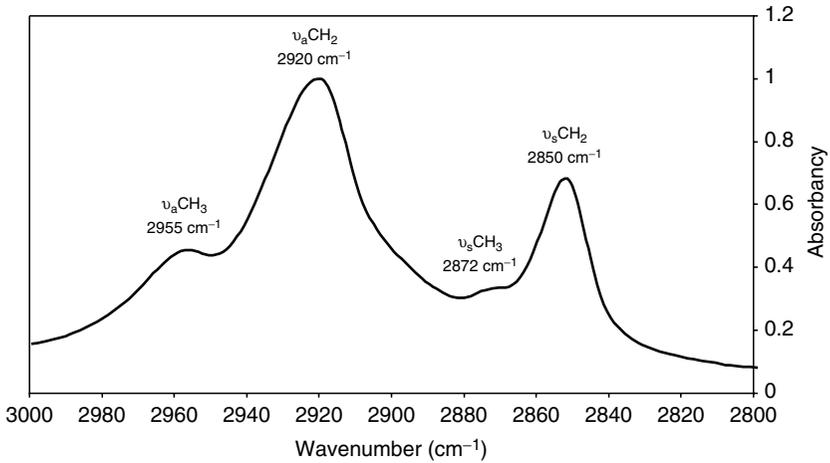
molecular mobility of water in the system compared with free water (Kuo *et al.*, 2001). It has been shown that measurement of the transverse relaxation ( $T_2$ ) time of protons allows the levels of water, fat or solids in dairy products to be estimated (Duce *et al.*, 1995; Chaland *et al.*, 2000).

## 20.2. Characterization of Dairy Products, Including Cheeses

### 20.2.1. Direct Determination of the Concentrations of Different Compounds

Reliable and rapid determination of quality parameters is an important problem for producers and marketers in the dairy industry. The proposed use of infrared reflectance spectroscopy to measure the composition of dairy products is based on the observation that the major components, fats, proteins and water, have absorption peaks in the near- and mid-infrared regions of the spectrum. The classical example of MIR for quantitation is the analysis of milk for payment, dairy herd recording or quality control (van de Voort *et al.*, 1987). Rodriguez-Otero and Hermida (1996) used NIR successfully to determine the fat content of fermented milk without any treatment of the sample. The same authors obtained better results when they analyzed the samples by near-infrared transreflectance spectroscopy. By legal definition, butter and margarine must meet minimum levels of fat and moisture. A Fourier-transform infrared (FTIR) method was developed to measure fat and moisture in butter (van de Voort *et al.*, 1992a). When butter samples were analyzed by the FTIR method, the results were within  $\pm 0.5\%$  of the values found by classical methods for each component. Total analysis time was  $\sim 5$  min/sample, providing results in time to allow a process operator to respond and adjust the manufacturing process in real time. The 3000–2800  $\text{cm}^{-1}$  region of the FTIR spectrum (Figure 20.2) also allows precise characterization of the fats in food products. FTIR makes it possible to determine rapidly and directly, iodine number, saponification number, *cis* and *trans* fatty acid content and free fatty acids (van de Voort *et al.*, 1992b, 1995; Ismail *et al.*, 1993). For example, C=C double bonds exhibit characteristic absorbance bands at about 3005  $\text{cm}^{-1}$  (*trans* isomer) and 3015  $\text{cm}^{-1}$  (*cis* isomer).

Recently, NMR has been applied to the analysis of mixtures. Analysis of the time-domain  $^1\text{H}$ -NMR signals from butter was performed using multidimensional statistical procedures. It has been shown that coupling FID, CPMG and inversion-recovery plots in a single matrix may, after statistical treatments, give information on the type and quantity of fat in the sample (Gil, 1997). The results obtained showed that NMR signals were



**Figure 20.2.** Mid-infrared spectrum of a cheese recorded in the 3000–2800 cm<sup>-1</sup> region.

more sensitive to changes in fat type than to changes in quantity. Signals from the protons of lipids were shown to provide more information on the type of fat, whereas signals from the protons of water were more sensitive to the fat content.

## 20.2.2. Direct Determination of the Structure of Fats in Dairy Products

### 20.2.2.1. Solid Fat Content of Fat Globules in Milk or Cream

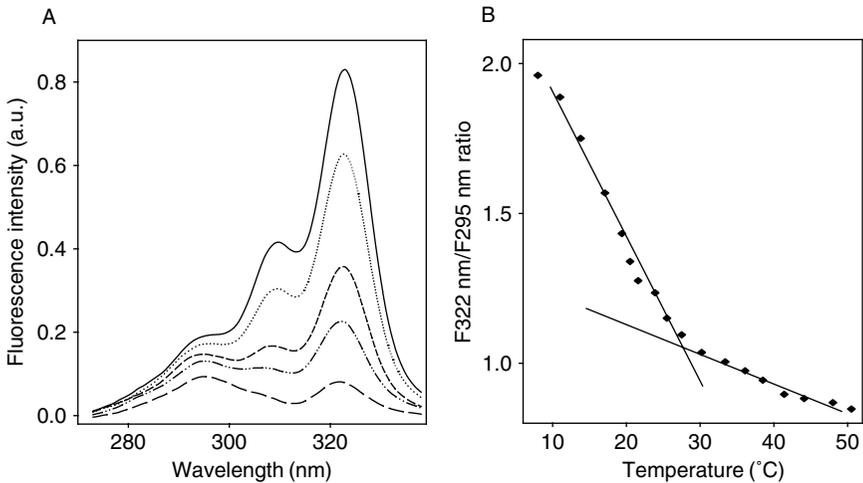
The lipids of milk fat globules contain hundreds of triacylglycerol species, the melting of which occurs over a wide temperature range (i.e., -30 to +40°C). Solid fat content is an important quality control parameter in the edible fats and oils industry. The organoleptic characteristics of creams, butters, margarines, cheeses and other fat blends depend partly on the value of this physical parameter. Determination of solid fat content has traditionally been based on dilatometry. Other commonly-used methods are nuclear magnetic resonance (Walker and Bosin, 1971) and differential scanning calorimetry (DSC). More recently, the value of infrared and fluorescence spectroscopies has been demonstrated (Dufour and Riaublanc, 1997b). These authors used the spectra of cream at various temperatures, ranging from 8 to 60°C, to evaluate the potential of FTIR spectroscopy for determining the solid fat content. NMR and DSC experiments were performed on the same samples and in the same temperature range. The

correlations observed between FTIR, NMR and calorimetric data indicated that there were relations between the changes in the absorbance at  $2865\text{ cm}^{-1}$  and the physical state of triacylglycerols in the temperature range investigated. Solid fat content can be predicted from differences in the absorbance in the MIR region due to the methylene group in solid or liquid fat (correlation coefficient = 0.996 and standard error = 0.62%).

It should be mentioned that DSC and NMR do not measure the same parameters, and in this way, these techniques are complementary. DSC is a dynamic method, which gives information about the transitions between different phases of lipids, whereas NMR allows quantitation of liquid and solid phases at equilibrium. Indeed, NMR and DSC methods give different values for the solid fat index (SFI) (Walker and Bosin, 1971; Norris and Taylor, 1977); NMR values are much lower than those given by DSC below  $20^\circ\text{C}$ . For example, for milk fat at  $5^\circ\text{C}$ , DSC and NMR indicate 78.1% and 43.7% solid fat, respectively. The observed difference can be explained by the presence of an amorphous phase which, due to its melting enthalpy, is seen as a solid by the DSC method. Using time-domain NMR, Le Botlan *et al.* (1999) showed that in milk fat samples, an intermediate component exists between the solid and liquid phases, constituting about 6% of an aged milk fat.

#### 20.2.2.2. Melting Temperature of Fats in Dairy Products

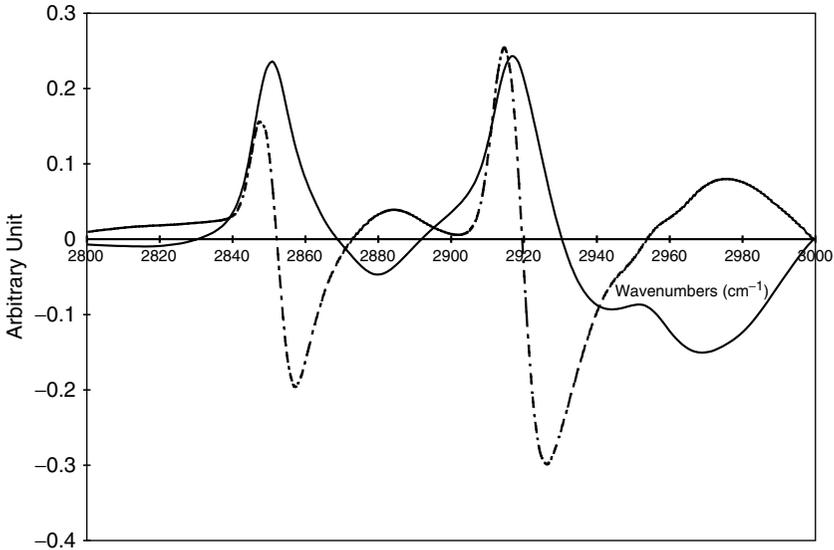
As mentioned above, fluorescent molecules are extremely sensitive to change in their environment, such as viscosity changes. For example, the emission of vitamin A is highly sensitive to its local environment, and is thus often used as a reporter group for viscosity changes in fat globules (Karoui *et al.*, 2003). The excitation spectra of vitamin A recorded between 250 and 350 nm, with the emission wavelength set at 410 nm (Dufour and Riaublanc, 1997a), provide information on the physical state of the triglycerides in fat globules (Dufour *et al.*, 1998). Indeed, the fluorescence spectrum of vitamin A in a milk fat-in-water emulsion stabilized by  $\beta$ -lactoglobulin showed changes when the temperature varied between 4 and  $45^\circ\text{C}$  (Figure 20.3A). The F322 nm/F295 nm ratio is modified by increasing the temperature (Figure 20.3B), allowing the melting point ( $28^\circ\text{C}$ ) of the triglycerides to be determined. It was shown that the solid fat content derived from fluorescence spectral data and from differential scanning calorimetry were similar. This result shows that the shape of the vitamin A fluorescence spectrum is sensitive to the physical state of the triglycerides in the fat globules of an emulsion. The melting temperature of fat has also been determined for cheeses using surface or front-face fluorescence spectroscopy (Karoui *et al.*, 2003).



**Figure 20.3.** Fluorescence properties of an emulsion at different temperatures. (A) Excitation spectra of vitamin A of a milk fat-in-water emulsion (melting point of fats = 28°C) recorded at 8.3 (—), 13.8 (···), 30.2 (— · —), and 49.1 (---) °C. (B) Changes in the ratio F322 nm/F295 nm with temperature.

### 20.2.2.3. Crystallization of Fats in Cheeses during Ripening

Texture is an important criterion used to evaluate the quality of cheese. It is generally assumed that texture of cheese changes with the physical state of its fat, depending on the storage temperature and time. Vitamin A fluorescence spectra collected directly on slices of 16 experimental semi-hard cheeses at 1, 21, 51 and 81 days of ripening allowed differentiation of the cheeses at the four different ripening times (Dufour *et al.*, 2000). Evaluation using Canonical Correlation Analysis (CCA) of fluorescence (vitamin A) and mid-infrared (2800–3000  $\text{cm}^{-1}$  region) spectra recorded on these 16 different semi-hard cheeses made it possible to understand better at a molecular level the structural modifications of lipids in cheeses occurring during ripening (Dufour *et al.*, 2000). The results of CCA performed on the two data sets showed that the first two pairs of canonical variables were correlated with a squared canonical correlation coefficient of 0.89 and 0.64, and that the similarity maps obtained allowed differentiation of the cheeses at the four different ripening times. Considering the mid-infrared region, the spectral pattern associated with the second canonical variate showed a shift towards lower values of the 2850 and 2920  $\text{cm}^{-1}$  bands from 21 to 81 days of storage (Figure 20.4). This pattern is characteristic of the liquid-to-gel phase transition (Casal and Mantsch, 1984; Dufour *et al.*, 1999). The results



**Figure 20.4.** Spectral patterns corresponding to the canonical variates 1 (—) and 2 (---) for FTIR data (Dufour *et al.*, 2000; see text for details).

obtained were consistent with a partial crystallization of lipids in cheese with time. Consequently, it has been proposed that the shift of the bands at 310 and 322 nm observed by Dufour *et al.* (2000) for the fluorescence spectral pattern associated with the second canonical variate is characteristic of the changes in lipid viscosity following partial crystallization. This result strongly indicates that the two spectroscopic methods make it possible to observe the structure of lipids in cheese at a molecular level.

### 20.3. Conclusion

Simple and rapid spectroscopic methods, such as front-face fluorescence, attenuated total reflectance Fourier-transform infrared and nuclear magnetic resonance spectroscopies, have a great potential for investigation of the structure of fats in dairy products and of the relation between structure and texture. Although fluorescence, infrared and NMR spectroscopies are techniques, the theory and methodology of which have been exploited extensively in studies in both chemistry and biochemistry, the usefulness of these spectroscopies for molecular studies has not been yet fully recognized in food science. Fluorescence, infrared and NMR spectroscopies coupled

with chemometry should be equally well suited to address the molecular structure of food products and the relations between structure and texture of dairy products.

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# Applications of Ultrasound to Analysis/Quantitation of Dairy Lipids

M.J.W. Povey and R.E. Challis

## 21.1. Introduction

Food quality is a complex amalgam of objective, measurable features and not-so-easily measurable consumer perceptions (Vickers and Wasserman, 1979). Clearly, no one technique, or indeed any ensemble of techniques, presently available, can measure all important aspects of food quality. So, the question regarding any technique under consideration for the measurement of quality is: “What can it usefully measure?”

From the general point of view, ultrasound has advantages regarding the measurement of dairy product quality in that it may be implemented in-line, non-invasively and even using non-contacting techniques such as laser excitation and detection (Mulet *et al.*, 1999). Ultrasound can also be safe, hygienic and economic in implementation, all characteristics desirable for any technique for the measurement of food quality (Povey, 1997a). Moreover, it can reveal aspects of the quality of dairy products which are not measurable by current techniques. An example is the extraordinary capability of ultrasound to detect crystal nucleation (Povey *et al.*, 2001; Hindle *et al.*, 2002).

Ultrasound is one of the more dynamic areas of food quality measurement, as indicated by the rapid rate at which commercial ultrasound instrumentation is entering the market (Mulet *et al.*, 1999; Povey, 2001; Povey and Higgs, 2001). Many reviews of the literature on the subject have also appeared recently (Povey, 1997a; Kress-Rogers, 2001). This dynamism is the result of two decades of developmental work (Povey and Wilkinson, 1980) in which ultrasound testing evolved from an empirical approach to

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

a model-based approach, together with developments in electronics and computing which make model-based instrumentation commercially feasible, as opposed to a laboratory curiosity. The principles upon which ultrasound measurement are based are, therefore, very important to successful measurement of food quality (Povey, 1997b). An empirical approach is generally undesirable in the analysis of ultrasound data because of the great number of variables upon which the ultrasound velocity and attenuation may depend. It is highly desirable to have a physical model, which can be used to relate ultrasound measurements to interesting properties of food. Otherwise, initially encouraging results may be confounded in practical application in ways which cannot be understood, undermining confidence in the technique.

It is claimed that commercially-available ultrasound equipment can measure the following quality parameters of dairy products: levels of solids, solids non-fat (SNF), protein, water and fat; solid fat content (SFC), colloidal stability, gelation point, adulteration with oil, particle size, particle size distribution, oil composition, protein denaturation and fat oxidation. This incomplete list represents an impressive contribution towards the solution of food quality measurement although the present authors are slightly skeptical regarding some of these claims. In this review only those applications will be addressed which are regarded as robust.

Foods, in general, present online physical measurements with quite a challenge, in particular the need to obtain 100% inspection arising from the highly variable nature of the raw materials. Ultrasound is one technique, which has the potential for 100% inspection using tomographic techniques (Beck *et al.*, 1994), in which the entire sample is penetrated by the inspecting field.

It is unlikely that ultrasound will be used on its own; rather, a range of techniques are likely to be brought to bear on process monitoring, fault detection and the achievement of a consistently high-quality product. Traditionally, this has focused on monitoring quality measurements, including sensory-based analysis, color analysis, measurements of texture and product consistency. More recently, online analytical measurements, such as NIR, Raman, NMR and ultrasound are used. These are combined with the routine collection of traditional process measurements such as temperature, pressure and flow. Integration of all these data will be the next stage in the development of process measurement.

In the following, the principles underpinning ultrasound measurement of food quality are discussed, online technologies are considered and applications examined, future trends identified together with sources of information.

## 21.2. Theory of Ultrasound Propagation Through Solid and Liquid Lipids

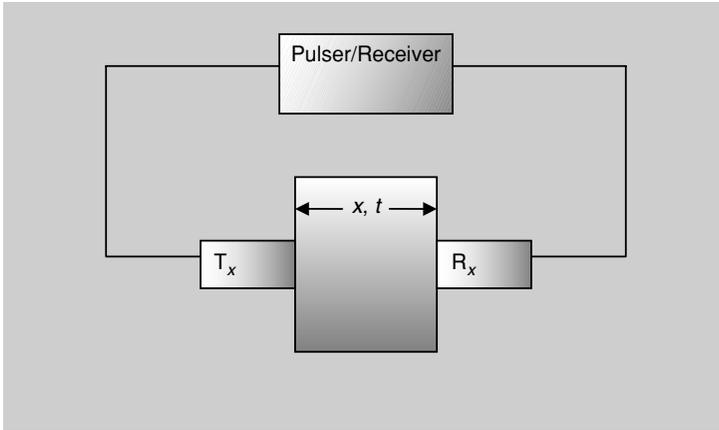
### 21.2.1. Introduction

When an ultrasonic wave propagates through a material (in this case, food) which is liquid, solid, or somewhere between the two, it does so at a certain speed known as *wave speed* or *propagation velocity*. This velocity and its (generally small) variation with frequency, or its variation with temperature may be used to characterize the material through which the wave has traveled. As the wave propagates, it may also lose energy to the surrounding material; this phenomenon is known as *wave absorption* or *attenuation*,  $\alpha$ . Again, the absorption that is measured depends on frequency,  $f$ , generally, but not always, rising as frequency increases; it also provides a means by which to characterize the material through which the wave has traveled. The wave at any distance,  $x$ , into a test medium can be described algebraically as

$$A(x,\omega) = A(0,\omega)e^{-\alpha(\omega)x}e^{-i\frac{\omega x}{c(\omega)}} \quad (1)$$

Where  $A(x,\omega)$  is the wave amplitude at position  $x$  and  $A(0,\omega)$  is the amplitude at the start of its journey in the test material.  $\omega$  is angular frequency,  $2\pi f$ , where  $f$  is in Hz.  $\alpha(\omega)$  is the absorption coefficient and  $c(\omega)$  is the phase velocity, both as functions of frequency,  $\omega$ . The first exponential term in Equation 1 thus gives a measure of energy absorption with distance, the decay in amplitude being exponential with respect to distance at any given frequency. The second exponential term expresses the phase change at frequency  $\omega$  as the wave travels to position  $x$  in the material. Note that the dimension of  $x/c(\omega)$  is that of time and  $\omega x/c(\omega)$ , therefore, represents the phase shift associated with propagation through distance  $x$ .

Figure 21.1 shows the configuration of a basic ultrasonic system. A generator excites the transmitting transducer,  $T_x$ , with a signal which can take many forms such as a short pulse, a sine wave burst, a chirp or even a noise-like signal, although the first two are most common. After propagating through the test medium, the wave excites the receiving transducer,  $R_x$ , from which it is amplified and in most systems, digitized for further processing. The propagation distance, also called *path length* or *gauge length*,  $x$ , may be variable or it may be fixed, particularly where there are physical constraints such as process pipework. The use of two transducers in this way is known as the *through transmission* or *pitch-catch* method. Under some conditions, the receiving transducer is eliminated and replaced by a wave reflector, which redirects the incident wave back to the transmitting transducer, which now doubles as both transmitter and receiver. This configuration is known as



**Figure 21.1.** Pulse-echo ultrasound configuration.

*pulse-echo* and is common in other applications of ultrasonics, such as medical imaging (Kremkau, 2001) and nondestructive testing (Krautkramer and Krautkramer, 1990; McSkimin, 1964). In the most simple systems, the speed of sound,  $c$ , is estimated from the time of arrival,  $\Delta t$ , of the signal after propagation through the test medium:  $c = x/\Delta t$ . However, it may not be possible to observe the precise time of arrival due to electronic noise masking the true start of the received signal. If  $A_T$  and  $A_C$  are the signal amplitudes received from the test and calibration materials, respectively, then the attenuation coefficient can be estimated to be:

$$\alpha = \frac{1}{x} \ln \frac{A_C}{A_T} \quad (2)$$

$\alpha$  is expressed in Nepers/m, where one Neper represents decay by the factor  $e^{-1} = 0.368$ , which is equivalent to  $-8.68589$  dB.

### 21.2.2. Physical Determinants of Attenuation, Phase and Group Velocity

The simplest, but not necessarily complete, description of a propagating wave is to be found in the one-dimensional wave equation, which is in essence a form of Newton's second law: *Force = Mass  $\times$  Acceleration*.

$$\frac{\partial^2 a(x,t)}{\partial t^2} = \frac{M}{\rho} \frac{\partial^2 a(x,t)}{\partial x^2}. \quad (3)$$

where  $\rho$  is the effective density of the material,  $a(x, t)$  is the instantaneous wave amplitude (pressure or displacement) expressed as a function of propagation distance and time, and  $M$  is the effective elastic modulus governing propagation. In all but the simplest materials at the lowest frequencies,  $M$  is a complex function of frequency.

$$M(\omega) = M_R(\omega) + iM_I(\omega) \quad (4)$$

where  $M_R$  and  $M_I$  are the real and imaginary parts, respectively, and  $i$  is  $\sqrt{-1}$ .

Using classical algebra (Bhatia, 1967, Matheson, 1971; McSkimin, 1964), phase velocity is given by:

$$c(\omega) = \left[ \frac{M_R(\omega)}{\rho} \right]^{1/2} \quad (5)$$

and attenuation is:

$$\alpha(\omega) = \frac{\omega}{2c(\omega)} \tan \delta(\omega) \quad (6)$$

where the effective loss tangent of the material is:

$$\tan \delta(\omega) = \frac{M_I(\omega)}{M_R(\omega)}$$

Three groups of phenomena affect the frequency-dependence of ultrasonic wave propagation: *classical processes*, *relaxation*, and *scattering*, of which scattering is likely to dominate in foodstuffs due to their particulate nature. The two classical thermal processes are radiation and conduction of heat away from regions of the material, which are locally compressed due to the passage of a wave; they can lead to attenuation but the effect is negligible in liquid materials (Herzfeld and Litovitz, 1959; Bhatia, 1967). The third classical process is due to shear and bulk viscosity effects. Attenuation in water approximates to a dependence on the square of the frequency and because of this it is common to express the attenuation in more complex liquids as  $\alpha(\omega)/\omega^2$  or  $\alpha(f)/f^2$  in order to detect, or differentiate from, water-like properties.

Relaxation processes occur when a wave propagates through a medium in which there exists any type of equilibrium, which is capable of being disturbed by the material state fluctuations associated with the passage of the wave. Detailed discussion of relaxation phenomena are to be found in

McCrum *et al.* (1967), Bhatia (1967) and Matheson (1971); their effects on ultrasonic absorption in solutions of amino acids and proteins have been studied by Holmes and Challis (1996) and Pavlovskaya *et al.* (1992 a, b, c).

Relaxation phenomena are commonly observed in homogeneous polymeric materials and are also one reason for the ability of ultrasound absorption to detect crystal nucleation (Akulichev and Bulanov, 1982; Povey, 1997b). Ultrasound velocity responds to crystal nucleation, mainly through the change in compressibility that occurs between the liquid and crystalline states (Povey, 1997b). Apart from the food sector, ultrasound has been shown to be able to differentiate between two crystal morphologies (Marshall *et al.*, 2000) and to monitor crystal growth (Povey, 1997b; Marshall *et al.*, 2002).

Most food materials are inhomogeneous in that they consist of particles suspended in a fluid or a solid matrix. Such particles act as scatterers of ultrasound even though their dimensions are generally much smaller than the ultrasound wavelength. For example, diagnostic ultrasound frequencies usually range from 1 to 100 MHz and in water the wave speed is around 1500 m/s, which gives wavelengths of 1.5 mm and 15  $\mu\text{m}$ , respectively, at these frequencies. We now consider the interactions of ultrasound with particulate mixtures, and we begin with a very fundamental measurement, namely that of adiabatic compressibility. It is perhaps surprising that sound velocity measurements are the only low-cost means to measure adiabatic compressibility. The basic relationship between sound velocity and compressibility was first established by Laplace as:

$$c = \frac{1}{\sqrt{\kappa\rho}} \quad (7)$$

This expression clearly has parallels with Equation 5 if we interpret adiabatic compressibility,  $\kappa$ , as the reciprocal of the elastic modulus,  $M$ . This simple equation is the key to an important class of food measurements because  $\kappa$  is related to changes in the tertiary structure of proteins, opening up the possibility of rapid characterization and the detection of denaturation (Aparenten *et al.*, 2000). For mixtures of materials, Wood (1941) invoked the idea of an *effective medium* to represent a mixture consisting of particles suspended in a continuum with compressibility and density given as:

$$\kappa_{\text{eff}} = \phi\kappa_1 + (1 - \phi)\kappa_2 \quad (8)$$

$$\rho_{\text{eff}} = \phi\rho_1 + (1 - \phi)\rho_2 \quad (9)$$

giving the speed of sound as:

$$c = \frac{1}{\sqrt{\kappa_{\text{eff}} \rho_{\text{eff}}}} \quad (10)$$

Here,  $\phi$  is the volume fraction of the dispersed phase, the properties of which are identified by subscript 1; subscript 2 represents the continuous phase. Although Equation 10 implies a homogeneous material and neglects scattering contributions from individual particles, it provides surprising and counterintuitive predictions. Equation 10 is sometimes attributed to Urick (1947), and Urick and Ament (1949), who used the effective medium idea to incorporate scattering from suspended particles. In the effective medium, average properties for density and compressibility are measured and attributed to the continuous and dispersed phase, respectively.

An elaborated version of the Urick equation derived from scattering theory and called the modified Urick equation (Pinfield and Povey, 1997) is :

$$\frac{1}{c^2} = \frac{1}{c_1^2} (1 + \delta' \phi + \delta'' \phi^2) \quad (11)$$

where  $\delta'$  and  $\delta''$  are scattering coefficients, which can be determined from experiment, by measuring sound velocity as a function of concentration, as well as being predicted from scattering theory. The homogeneous description for attenuation  $\alpha$  is given by :

$$\alpha = \phi \alpha_2 + (1 - \phi) \alpha_1 \quad (12)$$

Very accurate quadratic fits may be obtained from Equation 11, although Equation 12 is not so accurate and is not so well justified theoretically; however, it is the simplest available description. Note that the volume fraction,  $\phi$ , must be used and that this implies the use of partial molar volume and knowledge of molecular weight. Equation 11 works remarkably well and applies equally well to bovine serum albumin and casein.

It is very interesting that large molecules, such as proteins, behave as particles and can be described by ultrasound scattering theory in the very long wavelength limit. Scattering theory is vindicated by the precise and repeatable nature of the data available for these molecules. In particular, it should be pointed out that the molecular adiabatic compressibility is insensitive to individual bonds and is the sum of the intrinsic compressibility of the primary structure (the amino acid sequence), cavities in the tertiary structure and interaction with the solvent (Kharakoz and Sarvazyan, 1993). Velocity and attenuation spectroscopy relate to different aspects of the molecule

and the attenuation appears to be influenced by relaxation processes in the secondary structure (Povey, 1997b; Apenten *et al.*, 2000). Ultrasound spectroscopy has enormous potential for the rapid characterization of changes in protein structure, a potential yet to be realized.

There is an enormous volume of literature on the subject of acoustic wave scattering in particulate mixtures which will not be discussed here; the reader is referred to the literature which in terms of development of the subject is represented by Epstein and Carhart (1953), Ying and Truell (1956), Allegra and Hawley (1972) and Anson and Chivers (1993) and for multiple scattering by Foldy (1945), Waterman and Truell (1961) and Lloyd and Berry (1967). Together, this body of work is referred to as the Epstein–Carhart–Allegra–Hawley (ECAH) model. Later workers who have advanced the subject include Challis *et al.* (1998), O’Neill *et al.* (2001), and Povey (1997b). Suffice to say that the theory forms the basis of ultrasound particle sizing using ultrasound spectrometry.

#### 21.2.2.1. Detection of Flocculation and Networking

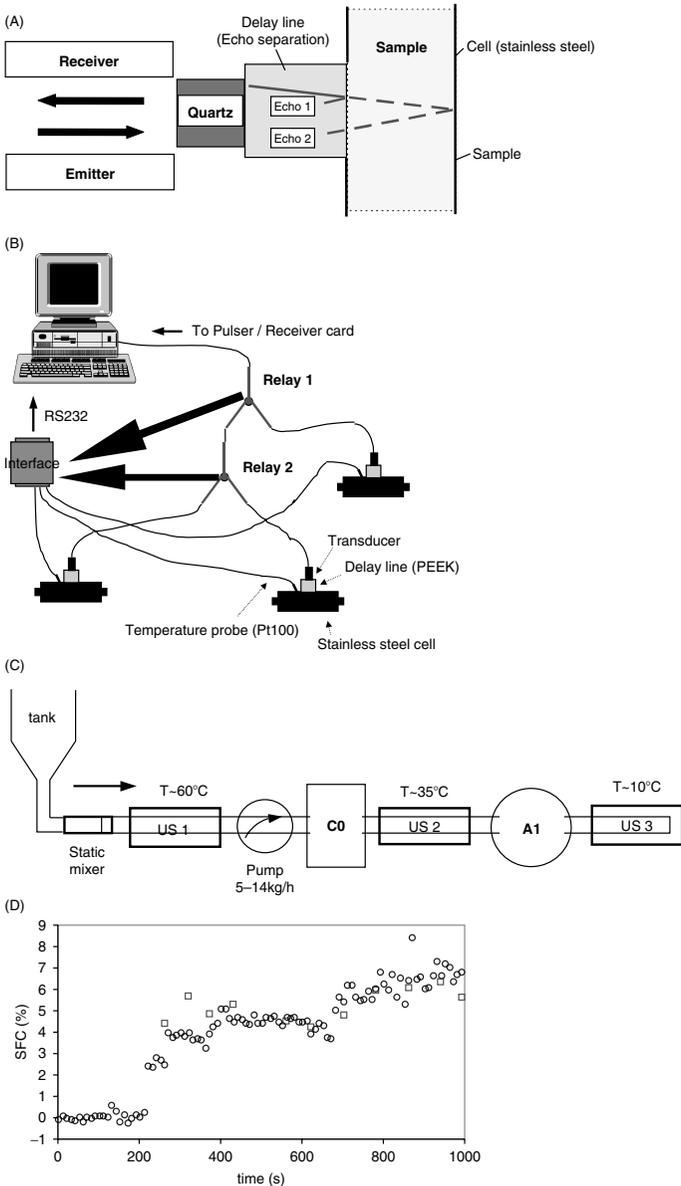
Many dairy emulsions destabilize by flocculation and networking. Hibberd *et al.* (1997a, b) obtained the ultrasonic response to flocculation showing that floc size increased during the experiment. The experiment was repeated with a higher level of hydroxyethyl cellulose (0.1%, v/v) with the result that flocculation occurred more rapidly with the formation of a densely connected network of particulate material. The residual root-mean-square error associated with fitting the ECAH model to the ultrasonic data at various points in the flocculation reaction increased rapidly at the onset of network formation and could, in principle, be used to detect such phenomena in a process context.

### 21.3. Ultrasound Methods for Characterizing Dairy Lipids

In a typical process, a transducer (transmit and receive) is mounted on a stainless steel pipe. Whilst the mechanical aspects of the construction of such systems are quite straightforward there is a very wide range of pipe sizes in a plant that requires monitoring. As an example, a system designed for use in a margarine plant is shown in Figure 21.2. Manufacturers of commercial equipment for ultrasound characterization of dairy lipids are listed in Table 21.1.

#### 21.3.1. Tracking Crystallization

Ultrasound is by far the most sensitive technique for detecting the initial stages of crystallization. Ultrasound velocity is very sensitive to the state of fat in a dairy emulsion (Figure 21.2) and can be used to follow changes in the amount of solid fat which occurs as the temperature changes.



**Figure 21.2.** Characterization of margarine using ultrasound. (A) In-line ultrasound configuration using a single transducer to monitor the solid fat content of margarine. (B) and (C) Multiple transducer configuration for following the evolution of solid fat content and (D) Time evolution of solid fat content measured by NMR of ultrasound velocity.

**Table 21.1.** Commercial equipment for ultrasound characterization of dairy lipids

Company	Device	Capability	Purpose	Currently Online?	Potential for in-line use
Cygnus Instruments, UK ( <a href="http://www.cygnus-instruments.com">http://www.cygnus-instruments.com</a> )	UVM1	Sound velocity	Materials characterization	Yes	Yes
Dispersion technologies, USA ( <a href="http://www.dispersion.com/">http://www.dispersion.com/</a> )	Scatterer Level detectors	Electro-acoustic spectroscopy Data interpretation software Pulse echo time of flight	Particle sizing, zeta potential Particle size analysis Level detection	No Yes	Yes Yes
Felix Alba Associates (Scatterer.com)	Ultrasizer	Attenuation spectroscopy	Particle sizing and materials characterization	No	Yes
KAB Instruments ( <a href="http://www.kabinstruments.com">www.kabinstruments.com</a> )	Acoustisizer	Electro-acoustic spectroscopy Multi capability	Particle sizing and zeta potential NDT	No	Yes
Malvern Instruments, UK ( <a href="http://www.malvern.co.uk">http://www.malvern.co.uk</a> )	UPR	Sound velocity	Milk analysis	No	Yes
Matec ( <a href="http://www.matec.com">www.matec.com</a> )	Alcohol meter	Sound velocity and density Pulse ultrasound instrumentation	Alcohol analysis	Yes	Yes
NDT solutions, UK ( <a href="http://www.ndtsolutions.com">www.ndtsolutions.com</a> )		Ultrasound attenuation Pulse echo time of flight	Particle sizing Concentration determination	Yes Yes	Yes Yes
Page & Pedersen, USA ( <a href="http://www.pagepedersen.com">www.pagepedersen.com</a> )		Attenuation and velocity spectroscopy-resonance	Materials characterization	No	Yes
Panametries ( <a href="http://www.panametries.com">www.panametries.com</a> )					
Sympatec ( <a href="http://www.sympatec.com">www.sympatec.com</a> )					
Rhosonics Ultrasonic Concentration Analysers ( <a href="http://www.rhosonics.nl">www.rhosonics.nl</a> )					
Ultrasonic Sciences ( <a href="http://www.ultrasonic-scientific.com">www.ultrasonic-scientific.com</a> )					

Ultrasound techniques to determine the solid content are accurate and precise methods for the determination of nucleation rates during isothermal crystallization, Gibbs free energy and the concentration of catalytic impurities (Hindle, 1996; Hindle *et al.*, 2000, 2002; Povey *et al.* 2001). The velocity of sound in an emulsion is measured while the sample is cooled rapidly and then held at a constant temperature. This temperature is varied in order to change the undercooling and hence the crystallization rate of the sample. The solid content is determined from the velocity of sound using the following equation (Povey, 1997b, 2001).

$$\phi = \frac{\frac{1}{c^2} - \frac{1}{c_1^2}}{\frac{1}{c_s^2} - \frac{1}{c_1^2}} \Phi_s \quad (13)$$

where  $\Phi_s$  is the total amount of solidifiable material in the dispersed phase.

Solid fat content (SFC) in fats is determined by on-line ultrasound measurement in which the transducers are bolted onto the pipe wall of the heat exchangers and crystallizers used to produce margarines (>80% fat content) and fatty spreads (>45% and <80% fat). The transducers use the pulse-echo mode in which the same transducer acts both as transmitter and receiver and the pulse bounces off the far wall of the pipe. In order to make accurate measurements, it is necessary to calibrate the system for particle scattering effects and this must be done prior to the initiation of crystallization. Firstly, the liquid line polynomial for the system must be established; this is the temperature-dependence of the speed of sound at temperatures above the melting point of the solid phase and determines  $c_1$  in Equation 13. Secondly, the solid line polynomial must be determined ( $c_s$  in Equation 13). This is not so easy because the temperature necessary to cause the complete freezing of the fat may lead to the freezing of the water phase. In this case, a semi-empirical equation may be used, which is adjusted to give the correct solid content (Povey, 1997b). Normally, measurements must be made at several points in the process and experience demonstrates that an accuracy of around 1% can be obtained; precision is around tenfold higher than the accuracy (Figure 21.2).

### 21.3.1.1. Developing Online Technologies

Measurement techniques for determination of sound velocity are given by Kuttruff (1991), Povey (1997b) and Asher (1997). In water, the

attenuation at 10 MHz is roughly 1.7 Np/m, (15 dB/m) whereas in oil-in-water emulsions it is not uncommon to find attenuations 120 dB greater than this (15 Np/m). These attenuations are the product of scattering. In addition, air bubbles, if present, may dominate the attenuation through resonant scattering.

It is far easier to measure sound velocity and attenuation in fluids than solids. The reason for this is that sample presentation is much simpler in the case of fluids because they flow around the transducers, which may be held static or even moved through the sample. This is not generally the case with solids. For an example of the in-line application of ultrasound to the measurement of solid fat content in margarine during manufacture, see Figure 21.2.

### 21.3.1.2. Online Determination of Water Content

The water content (and hence the oil content) of emulsions may be obtained by measuring the temperature dependence of the velocity of sound. Firstly, it is necessary to melt any crystallized fat by heating it above its melting point. In most food emulsions, such as milk, this requires heating to 40°C when all of the fat will be melted. An equation similar to 13 gives the water content,  $\phi_w$  from a measurement of the temperature coefficient of the velocity of sound:

$$\phi_w = \frac{\frac{1}{v^3} \frac{dv}{dT} - \frac{1}{v_{oil}^3} \frac{dv_{oil}}{dT}}{\frac{1}{v_w^3} \frac{dv_w}{dT} - \frac{1}{v_{oil}^3} \frac{dv_{oil}}{dT}} \quad (14)$$

In the case of milk, it is possible to take this further. The temperature coefficient of the velocity of sound is well known in both oil and water, so the water content can be obtained from the measured temperature coefficient of the velocity of sound in milk above 40°C where all the fat is liquid; the oil content can be obtained by difference. With knowledge of the levels of water and oil, the protein content can be estimated from the absolute value of the speed of sound. The protein content so determined will be influenced by the location and state of the protein (micelle state or at the oil/water interface) arising from processing of the milk and also by other factors such as lactose and salt.

## 21.4 Conclusions

Ultrasound measurement will undoubtedly be used in the future in the dairy industry. However, it is not yet fully clear which applications are likely to be important. Some manufacturers claim that rheological measurements

may be made from ultrasound velocity and attenuation spectra. It is important to emphasize that the ultrasound velocity and attenuation spectra are not directly related to rheological measures such as the real and imaginary parts of the rigidity modulus (Povey, 1997b). In fact, it is shown above that these quantities are related to the total modulus,  $M$ , of which the rigidity modulus,  $G$ , is perhaps  $10^8$  times smaller than the bulk modulus,  $K$ , for most foods. The correlations found between  $v(f)$  and  $\alpha(f)$  arise due to changes in the scattering arising as particles associate and flocculate in the process of forming gels. Since this is the same process which gives rise to gels, a correlation may be observed between  $G$ ,  $\alpha(f)$  and  $v(f)$ . This does not invalidate the use of ultrasound to follow some gelation processes, but it is incorrect to say that these direct measurements actually measure  $G$ .

It is already clear, however, that ultrasound can provide unique information regarding crystallization processes and as the technology and underlying data interpretation models improve it is likely to make a very significant contribution to improving the quality of dairy products. In particular, better understanding of the interaction of ultrasound fields with proteins is required.

## Acknowledgements

The authors gratefully acknowledge permission by Unilever (Dr Geert van Kempen) to reproduce Figure 21.2.

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# Physical Characterization of Milk Fat and Milk Fat-Based Products

O.J. McCarthy

## 22.1. Introduction

The sensory properties, especially texture and appearance, of milk fat-based products such as butter, cream, cheese, ice cream and milk chocolate are largely dependent on the physical properties of the product, especially properties governed by the *phase change behavior* of the fat, used here to mean melting and crystallisation behavior, crystal polymorphism and microstructure (Birker and Padley, 1987; O'Brien, 2003). The same may be said of the functional properties of milk fat, milk fat fractions and milk fat-based products when these are used as food ingredients.

The term *physical properties* is used here to mean both physical properties as such (e.g., density) and characteristics that can be measured by physical means (e.g., polymorph type). The measurement of physical properties is essential for the proper understanding and control of processing operations and of the sensory properties, functionality and shelf stability of the final product (Birker and Padley, 1987; Rajah, 2002; O'Brien, 2003). Techniques for measuring phase change behavior are described first. These are grouped, somewhat loosely, under the heading *thermal properties*, together with techniques for measuring milk fat critical temperatures. Second, the measurement of *rheological properties* and of *density* is described under these headings. Then, methods for measuring refractive index, color, dielectric properties and electrical conductivity are described under the heading *electromagnetic properties*. These headings are not mutually exclusive. Optical methods for measuring phase change behavior are included under *thermal properties* rather than under *electromagnetic properties*. Rheological methods of particular use for

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*Advanced Dairy Chemistry, Volume 2: Lipids, 3rd edition.*

Edited by P.F. Fox and P.L.H. McSweeney, Springer, New York, 2006.

measuring phase change behavior are also included under *thermal properties*, but are described in more detail under *rheological properties*.

The last main section, *functional properties*, comprises descriptions of techniques for measuring “ingredient properties” and “end use properties” of milk fat and milk fat-based products.

The measurement techniques described in this chapter are those of greatest importance and utility for the physical characterization of milk fat and milk fat-based foods for research and food industry purposes.

## 22.2. Thermal Properties: Phase Change Behavior

### 22.2.1. Melting and Solidification Points: Introduction

Milk fat is a complex and variable mixture of triacylglycerols (TAGs). Each of these, and each polymorph of a given TAG, has its characteristic melting point. The full range of TAG melting points is approximately  $-40$  to  $72^{\circ}\text{C}$ . However, the melting point range of milk fat itself is about  $-40$  to  $37^{\circ}\text{C}$ , because higher melting point TAGs dissolve in the molten lower melting point TAGs (Rossell, 2003).

Determinations of milk fat melting and solidification points are attempts to quantify the upper end of the milk fat melting point range. The result obtained from a measurement depends on the method used; a melting point or a solidification point is defined by the technique and conditions of measurement, and the reported value is specific for these (Kaylegian and Lindsay 1994; Firestone, 1998).

### 22.2.2. Melting Points

#### 22.2.2.1. Clear Point

The clear point, also called the *clear melting point*, *complete melting point*, *complete fusion point* or *capillary melting point*, is the temperature at which a sample of fat becomes visibly completely clear, indicating the disappearance of all traces of solid fat (Rossell, 1986; Stauffer, 1996). Its measurement is specified by the American Oil Chemists’ Society (AOCS) Official Method Cc 1–25 (Firestone, 1998). Samples of the tempered fat contained in at least three vertical glass capillary tubes, sealed at their lower ends, are attached to a vertical mercury-in-glass thermometer such that their lower ends are level with the lower end of the thermometer’s bulb. This assembly is immersed in a water bath and heated, and the

temperature at which the sample in each capillary becomes clear is observed. The clear point is taken as the mean of these temperatures.

This measurement is claimed to be more reproducible than the slip point (see below), and is the most widely accepted technique for determining melting point (Rossell, 1986; Kaylegian and Lindsay, 1994). It is the method usually used for milk fat.

Clear points can be determined objectively by dilatometry, differential scanning calorimetry or nuclear magnetic resonance (Mertens, 1973; deMan *et al.*, 1983). Measured values are not necessarily equivalent either to that obtained using the AOCS Cc 1–25 standard method or to each other. Dilatometry and differential scanning calorimetry are described below.

#### 22.2.2.2. Slip Point

The slip point, sometimes called the *slip melting point*, *softening point*, *rising point* or *open tube melting point* (Rossell, 1986; Firestone, 1998; O'Brien, 2003) is measured using a technique essentially identical to the capillary tube method for clear point (AOCS Cc 1–25), except that the tempered fat is contained in capillary tubes open at both ends, and the temperature at which the fat column begins to rise (slip upwards) in each tube is observed. The slip point is taken as the mean of these temperatures. Details of the basic method are given in AOCS Official Method Cc 3–25 (Firestone, 1998). A more sophisticated version of the test, based on International Standard ISO 6321, is described in AOCS Official Method Cc 3b–92 (Firestone, 1998). This version has two alternative procedures, one applicable to fats solid at room temperature that do not exhibit polymorphism, and the other applicable to all fats solid at room temperature. The second procedure is probably the best way for determining the slip point of milk fat and milk fat fractions.

The slip point is lower than the clear point and corresponds to a solid fat content of 4–5% (Rossell, 1986; Stauffer, 1996). The slip point of fats can be used as an indicator of relative differences in melt-in-the-mouth characteristics (Mertens, 1973).

Slip points are commonly measured for vegetable fats, while clear points are usually measured for milk fat. Care should therefore be taken when comparing reported melting points for these two kinds of fat (Rossell, 2003).

#### 22.2.2.3. Dropping Point

The dropping point, commonly called the *Mettler dropping point*, is defined as the temperature at which a fat becomes sufficiently fluid to flow

under the conditions of the test (Firestone, 1998). It is measured using a proprietary apparatus made by Mettler–Toledo, Inc (Greifensee, Switzerland). In contrast to the clear point and slip point techniques described above, the measurement is objective and automatic.

The molten fat sample is allowed to solidify in a cold sample cup, or a finished product such as butter is placed in the cup, with the cup standing on a clean smooth surface. The assembly is then allowed to stand in a freezer at  $\leq -5$  °C for at least 15 min. Then, the cup, which has a small hole in its base, is placed in the furnace of the Mettler apparatus. The temperature of the furnace is ramped upwards automatically. When the fat becomes fluid enough to flow, a drop of partly molten fat falls from the hole in the cup, tripping a photoelectric circuit by temporarily blocking a light beam that keeps the circuit closed. The temperature of the furnace at which this occurs is displayed automatically as the dropping point (Kaylegian and Lindsay, 1994). Details of the technique are specified in AOCS Official Method Cc 18–80. A subjective version of this test is described in British Standard BS 684: Section 1.4: 1976 (BSI, 1976a).

As the dropping point method can be applied to fat fractions and fat-based ingredients, as well as to finished products made from them, it provides a means of studying the influence of manufacturing conditions (Mertens, 1973).

The dropping point corresponds to a solid fat content of about 5% (Papalois *et al.*, 1996), and corresponds closely to the softening point determined by the method of Barnicoat (1944) (Timms, 1978; deMan *et al.*, 1983; Papalois *et al.*, 1996). Barnicoat's method was developed for application to milk fat fractions as an alternative to the tedious Wiley melting point technique (Mertens, 1973). In Barnicoat's method, the softening point is taken as the temperature at which a steel ball bearing, initially on the surface of a column of the fat, sinks halfway through the column. In the Wiley method, historically favored in the US, but now an AOCS "surplus" official method (Firestone, 1998), the melting point is taken as the temperature at which a disc of initially solid fat located at the interface between a layer of water and an (upper) layer of 95% ethanol, becomes spherical. The Barnicoat method is still used in the chocolate industry (Minifie, 1999).

Nakae *et al.* (1974) used a temperature gradient apparatus to determine the melting point of butterfat samples as the temperature at the boundary between the solid and liquid parts of the sample. Values obtained were correlated with melting points determined by two conventional methods (unidentified in the English summary of this Japanese-language paper).

References to the literature on the measurement of the melting points of milk fat fractions are tabulated by Kaylegian and Lindsay (1994).

### 22.2.3. Solidification Points

#### 22.2.3.1. Cloud Point

The cloud point is defined as the temperature at which a molten fat sample becomes cloudy, owing to the appearance of fat crystals, while being cooled. Versions of the test, which is a simple subjective one, are described in AOCS Official Method Cc 6–25 (Firestone, 1998) and in British Standard BS 684: Section 1.5: 1987 (BSI, 1987). As supercooling is necessary to induce fat crystallization, the cloud point is always lower than the clear point (Kaylegian and Lindsay, 1994).

#### 22.2.3.2. Congeal Point

The congeal point, also called the *setting point* (O'Brien, 2003), is a measure of the solidification point of a molten fat under the conditions of the test. The molten fat, contained in a beaker, is cooled until the cloud point is observed, and then cooled further until a certain subjectively assessed degree of turbidity (caused by the presence of fat crystals) is reached. The beaker is then kept at 20°C and the temperature of the sample recorded over time. The temperature rises initially owing to the release of latent heat of crystallization and then drops. The maximum temperature reached is recorded as the congeal point.

### 22.2.4. Dilatometry

Dilatometry is a technique for measuring the solid fat index (SFI), which is an estimate of the mass fraction of a fat that is solid at the temperature of measurement.

The *dilation* (or dilatation) of a partly solidified fat sample at a given temperature is the increase in specific volume (volume per unit mass) that occurs during isothermal complete melting at that temperature. The *melting dilation* of the fat at the same temperature is the increase in specific volume that would occur on complete melting if the sample was initially 100% solid. Dilation is the result of the expansion that occurs as a result of the solid to liquid phase change; the specific volume of molten fat is about 10% greater than that of solid fat.

The quantity

$$\left( \frac{\text{dilation}}{\text{melting dilation}} \right) \times 100\%$$

is an estimate of the solid fat content by mass percentage (SFC). It is called the *solid fat index* (SFI). The relationship between SFI and SFC is a function of fat type, temperature and SFI level (Stauffer, 1996).

Dilation is measured using a specialized pycnometer, called a dilatometer. The conventional dilatometer is a glass apparatus consisting of a vertical bulb, equipped with a stopper, connected by a U-tube to a vertical capillary tube graduated in microlitres. A volume of aqueous dye solution (or mercury for very precise measurements) is placed in the bulb. A suitable volume of the molten fat sample is then poured into the bulb to float on the dye solution, pushing a portion of the dye solution into the capillary (Lewis, 1987). The weight of the fat sample is determined by weighing the dilatometer before and after loading it with the sample and inserting the stopper.

Volume measurements needed to calculate the SFI are made by observing and recording the position of the dye solution meniscus in the graduated capillary tube at one temperature (typically 60°C) or two temperatures at which the sample is fully molten, and at desired measurement temperatures (which will be lower than the clear point of the sample). The sample is brought to the first measurement temperature from 0°C after a standardized tempering procedure. This and subsequent measurement temperatures must be approached from below to avoid supercooling effects. Temperature is controlled by immersing the whole dilatometer in constant temperature water baths or an ice-water bath.

The interpretation of dilatometric volume measurements is complicated by the fact that the molten fat has a coefficient of thermal expansion (about 0.00084 mL/gK) more than twice that of solid fat (about 0.00038 mL/gK). This difference means that the melting dilation is temperature-dependent (Stauffer, 1996). In principle, this could be allowed for by measuring the specific volume of both the fully molten fat and the fully solid fat at the measurement temperature. However, while the specific volume of the molten fat is easily measured using the dilatometer, that of the solid fat is not. This difficulty is circumvented by assuming, by convention, that the temperature dependence of specific volume is the same for both the molten fat and the solid fat (thus making the melting dilation temperature independent), and that the melting dilation itself has a constant value, independent of fat type, of 0.1 mL/g (= 0.1 L/kg). Thus (Hannewijk *et al.*, 1964),

$$\text{SFI} = \frac{\text{dilation (mL/g)}}{0.1} \times 100\% = \frac{\text{dilation(mL/g)}}{0.001} \quad (1)$$

Dilatometer specifications, and measurement procedures and calculations, are published in the form of standards by the AOCS (AOCS Official Method Cd 10-57, Firestone, 1998), the International Union of Pure

and applied chemistry (IUPAC standard method 2.141, Paquet and Hautfenne, 1987) and the British Standards Institution/International Organisation for Standardization (ISO) (BS 684: Section 1.12:1990/ISO 8293:1990, BSI, 1990). Standard methods incorporate implicit measurement of the temperature dependence of the specific volume of the molten fat, and corrections for the thermal expansion of the dye solution and of the dilatometer itself.

The theory and principles of dilatometry are particularly well described by Hannewijk *et al.* (1964).

Dilatometry is accurate and cheap, but time-consuming and laborious (Wan, 1991; Kaylegian and Lindsay, 1994; Shukla, 1995). The technique was accepted worldwide up to about 1970, but a switch to the use of nuclear magnetic resonance (NMR) began in the early 1970s (Shukla, 1995).

### 22.2.5. Nuclear Magnetic Resonance

Pulsed nuclear magnetic resonance (pNMR) has become the preferred method for measuring the percentage of solid fat in a partly solidified fat sample in most parts of the world because of its speed, precision and utility (Kaylegian and Lindsay, 1994). In contrast to dilatometry, pNMR measures the true solid fat content (SFC) rather than an index of this.

Workers in the United States have been slow to make the switch from dilatometry to pNMR (Shukla, 1995). However, the AOCS has published two standard methods for determining SFC by, respectively, pNMR and low-resolution NMR (AOCS Official Methods Cd 16–81 and Cd 16b–93, Firestone, 1998).

The measurement of SFC by pNMR is commonly used to monitor fat crystallization. It is, however, generally less sensitive than absorbance spectrophotometry (see below) in the early stages of crystallization, as crystals can be visible before solid fat is detectable by pNMR (Wright *et al.*, 2001a). Notwithstanding this, Wright *et al.* (2000) found a strong correlation between the induction time measured by pNMR and that measured by absorbance spectrophotometry for three milk fat systems. The principles of NMR are described in Chapter 20.

### 22.2.6. Differential Scanning Calorimetry

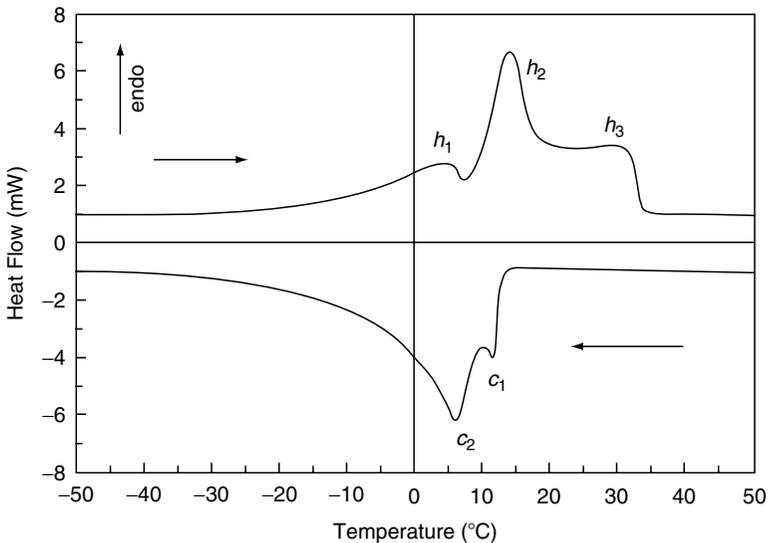
Differential scanning calorimetry (DSC) is a technique for measuring the thermal behavior of a substance. In DSC, the difference between the heat flow (J/s, or W) to or from a sample and a reference material is measured as a function of temperature or time while the sample and the reference material are subjected together to a controlled

temperature-time programme (Wright, 1984). DSC is thus a form of differential thermal analysis (DTA).

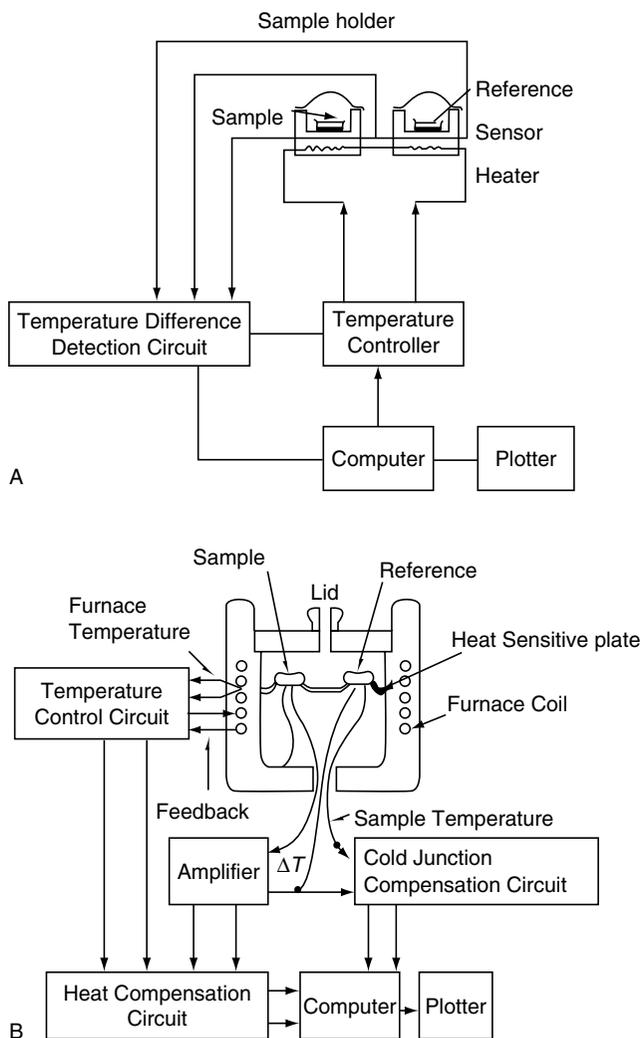
The reference material is chosen to be one that exhibits no thermally induced transitions within the temperature range of interest. Thus, transitions that occur in the sample during the applied temperature programme appear as peaks or troughs, depending on whether they are exothermic or endothermic, on the plot of differential heat flow versus temperature (the thermogram) that is the output of the instrument (Figure 22.1). Commonly used reference materials are listed by Hatakeyama and Quinn (1994).

In the context of milk fat, DSC is most widely used to measure temperatures and heats of transitions (phase changes). It is also used to measure specific heat, solid fat content, crystallization kinetics constants and fat purity, and in the study of fat crystal polymorphism.

Most commonly used DSC instruments fall into two categories (Laye, 2002): power compensation (PC) instruments, for which the term DSC was coined when this type became available in 1963 (Wendlandt, 1974), and heat flux (HF) instruments (Figure 22.2). The latter are essentially quantitative DTA instruments; classical DTA is a qualitative, or at best semi-quantitative, technique (Wright, 1984).



**Figure 22.1.** Typical DSC thermogram for milk fat.  $c_1$  and  $c_2$  are exothermic crystallization peaks obtained during cooling.  $h_1$ ,  $h_2$  and  $h_3$  are endothermic peaks obtained during heating. (Reproduced with permission from ten Grotenhuis *et al.*, 1999.)



**Figure 22.2.** Schematic diagrams of (A) a power compensation DSC and (B) a heat flux DSC. Heat sensitive plate: heat flux plate. (Reproduced with permission from Hatakeyama and Quinn, 1994.)

In any DSC instrument, the sample and reference material are placed in small individual pans or crucibles, which may be open or hermetically sealed, of 10–20  $\mu\text{L}$  capacity (Harwalkar and Ma, 1990). Sample size is

therefore small, and accurate weighing is essential. Sometimes, the reference is merely an empty pan.

In PC instruments (Figure 22.2A), the sample and reference pans are placed in individual holders (sometimes called furnaces), each equipped with an electric heater and a temperature sensor. During a temperature scan, the sample and reference are maintained at exactly the same temperature at any instant, even when a thermal event (transition) is occurring in the sample (Daniels, 1973). This null temperature balance is achieved by automatic adjustment of the power supplied by the heater in the sample holder (Daniels, 1973). Adjustment occurs when the temperature difference between the sample and reference holders greater than a set threshold value (typically  $<0.01$  K) is detected (Hatakeyama and Quinn, 1994). The instrument output signal is the difference between the power supplied to the sample holder and that supplied to the reference holder. The former is higher during an endothermic transition, and lower during an exothermic transition.

In HF instruments (Figure 22.2B), the sample and reference pans are heated or cooled in a single temperature-controlled furnace. In the most common design, the two pans rest on a horizontal metal plate “the heat flux plate” connected directly to the heating block of the furnace. The heating block is of large thermal mass and often made of silver or other noncorroding metal of high thermal conductivity. The temperature of the block is programmed as required, the good heat flow path provided by the heat flux plate ensuring that the sample and the reference are subjected to the same programme.

When a thermal event occurs in the sample, a temperature difference tends to develop between the sample and the reference. This results in a heat flux between the two *via* the heat flux plate. This heat flux ensures that the temperature difference between the sample and the reference is always very small. This is important for ensuring that both sample and reference are subjected to essentially the same temperature programme (Bhadeshia, 2002).

However, it is this small temperature difference between sample and reference that is measured as a function of temperature during the applied temperature scan. It is converted by the instrument’s software, using instrument calibration data, to the difference between the power absorbed or released from the sample and that absorbed or released from the reference. The final instrument output is a thermogram of this differential power plotted against temperature, as for PC instruments.

At least one DSC instrument is available that incorporates features of both PC and HF types (Laye, 2002).

When DSC scans are carried out below room temperature, or when the temperature programme is one of decreasing rather than increasing temperature, controlled cooling rather than heating of the sample and reference

is required. This is usually achieved by means of an efficient cooling system (built into the instrument) the cooling effect of which is moderated by input of electric power *via* the heater (HF) or heaters (PC) to give the cooling rate required (Daniels, 1973; Wunderlich, 1990; Laye, 2002).

Bashir *et al.* (1998), in a study of phase transitions in a monotropic liquid-crystal polyester, found that PC and HF instruments performed differently in cooling mode. Better resolution of thermal events (i.e., better separation of peaks on the thermogram) was obtained with PC instruments. These authors pointed out that the performance of any DSC instrument might not be the same during heating as during cooling.

Conventionally, the temperature programme used in DSC, whether heating or cooling, is a linear change in temperature with time. Isothermal DSC, in which the temperature is kept constant at a value at which a transition of interest is known to occur, is especially useful for studying the crystallization of a fat (Simon and Süverkrüp, 1995; Blaurock, 2000). In this technique, the sample (after holding for a time in a fully molten state to erase thermal memory) and the reference are cooled rapidly to the sample crystallization temperature and, after temperature equilibration, the differential power input is measured as a function of time under isothermal conditions. The heat of crystallization released by polymorphs in the fat sample results in exothermic signals, which appear as peaks on the thermogram. The nucleation time (the time at which a peak starts forming), time of maximum crystallization rate (the time of peak maximum) and heat of crystallization (proportional to peak area) can all be determined from the thermogram.

A DSC heating thermogram for the same fat sample can conveniently be measured as soon as isothermal crystallization has gone to completion.

Also, after determining the temporal distribution of crystallization peaks in a complete isothermal experiment, the melting thermogram of a particular polymorph can be determined. First, the sample is remelted completely, and held to erase its thermal memory. Then, the isothermal crystallization process is repeated, but only up to the time at which the polymorph of interest has completely solidified (exothermic peak fully formed). A heating scan is then performed immediately (Kawamura, 1980, 1981).

The effects of different rates of cooling down to isothermal crystallization temperature, different crystallization temperatures and different rates of heating during melting, on polymorph formation and transformation are easily studied by DSC. However, it is not possible unequivocally to identify polymorphs by DSC; this must be done by X-ray diffractometry (Rossell, 2003; see below).

Although isothermal DSC is conceptually a good technique for studying fat crystallization, some workers (e.g., Wright *et al.*, 2001a) have found it

inferior to other techniques such as pNMR, turbidimetry, light-scattering and polarized light microscopy (see below) for studying the crystallization of milk fat. The reason for this was said to be an inherent lack of DSC instrument sensitivity at the high cooling rates needed to maintain isothermal conditions during crystallization.

Breitschuh and Windhab (1996) described a novel use of DSC for directly investigating or monitoring crystallization processes such as those used in the fractionation of milk fat. In their technique, called "DSC direct," a small sample of fat is taken from the process line, placed immediately in the sample pan of the DSC instrument, and within seconds, subjected to a heating scan. In this way, the melting characteristics of the fat at that particular stage of the process, as determined by processing conditions up to that stage, can be determined directly.

PC instruments are preferable to HF instruments for isothermal studies, and in studies in which the temperature scanning rate is high, because the very small sample and reference holders in the PC type have much smaller thermal inertia than the relatively large heating block in the furnace of the HF type (Hatakeyama and Quinn, 1994).

More complex temperature programmes are sometimes useful. These might combine periods of variable heating and cooling rates with isothermal periods. For example, stepwise heating can be used to detect the onset of melting under quasi-isothermal conditions (Laye, 2002). Modulated temperature DSC (MTDSC), in which the linear temperature scan is perturbed by a sinusoidal, square or saw-tooth wave, or other modulation of temperature, has a number of potential advantages over the conventional linear scan. These include increased sensitivity and resolution, and the ability to separate multiple thermal events (Laye, 2002).

High sensitivity DSC (HS-DSC) instruments are used to measure small heats of transition. They were developed originally for measuring to heat of denaturation of biopolymers in dilute solution (Hatakeyama and Quinn, 1994). The sensitivity of a heat flux instrument, for example, can be improved by increasing sample size, using multiple serially-connected thermocouples to measure both sample and reference temperatures, and increasing the heat sink capability of the heat flux plate (Hatakeyama and Quinn, 1994).

As already stated, the usual output of a DSC instrument is a thermogram. This is a plot of differential heat flow rate (differential power) versus temperature for a temperature scan, or a plot of differential heat flow rate versus time for an isothermal scan. The thermogram is most logically plotted (by the instrument's software) such that a peak represents an exothermic event in the sample while a trough represents an endothermic event. However, the thermogram from a PC instrument is sometimes plotted in the

opposite sense; this is not entirely illogical since in this form an exothermic event results in the quantity (*power supplied to sample* – *power supplied to reference*) being negative during an exothermic event and positive during an endothermic event. The direction of exothermic and endothermic events should always be indicated on the ordinate of the thermogram.

The instrument software can be used to derive properties of the sample from the thermogram data. These may include specific heat capacity, temperature of transition, heat of transition, solid fat content and reaction kinetics constants.

#### 22.2.6.1. Specific Heat Capacity

When a linear temperature scan has been used, the ordinate of the thermogram can be converted from differential power to sample specific heat capacity if the mass of the sample is known (Wright, 1984). The variation of specific heat capacity with temperature is then easily determined. When the temperature of determination falls within a range in which phase transition is occurring, the measured specific heat capacity is an apparent one, as sensible and latent heat changes are then confounded. Usually, specific heat capacity is measured by DSC relative to that of a standard material such as sapphire for which very accurate specific heat capacity data are available (Hatakeyama and Quinn, 1994). This avoids inaccuracy owing to inherent errors in the range calibration and programmed heating rate of the DSC instrument (Wright, 1984).

#### 22.2.6.2. Temperature and Heat of Transition

DSC is a particularly useful technique for measuring temperature and heat of transition, two properties of considerable importance with respect to the thermal behavior of fats.

The properties are determined for a given transition by suitable analysis of the corresponding peak (or trough) on the thermogram. The peak temperature is easily identified but is a less suitable measure of the transition temperature than the onset temperature, the temperature at which transition begins. The onset temperature can be difficult to determine as the thermogram curve often departs only very gradually from the baseline, the path the curve would follow if no transition occurred. A number of mathematical strategies are available for dealing with this problem (Wright, 1984).

The heat of transition is found by measuring the area under the peak of the thermogram, to which it is directly proportional:

$$\text{Heat of transition} = K \times \text{peak area} \quad (2)$$

where  $K$  is an instrument calibration constant established empirically using standard materials with accurately known heats of fusion. Standards commonly used are listed by Hatakeyama and Quinn (1994).

Determination of the peak area is complicated if the specific heat capacity of the sample changes over the temperature range of the transition, as the form of the baseline is then not known. Approaches to dealing with this problem are described by Daniels (1973) and Wright (1984).

#### 22.2.6.3. Solid Fat Content

A thermogram obtained by completely melting an initially completely solid fat sample using a linear temperature ramp can be used to determine the relationship between solid fat content and temperature. The baseline is assumed to be a straight line between the upper and lower limits of the melting temperature range (Norris *et al.*, 1971). The solid fat content at a given temperature within the melting range is calculated as the ratio of the area under the curve between that temperature and the upper limit to the total area. Selection of the lower melting limit is difficult because of the small slope of the initial part of the melting curve, but error in this selection does not greatly affect accuracy (Norris *et al.*, 1971). This technique is an alternative to dilatometry and NMR. However, though rapid, its precision and reproducibility are less than those of dilatometry, high sample weighing accuracy is required, and in fact too much information is produced. Thus, the technique appears to be unsuitable as a routine method for measuring solid fat content (O'Brien, 2003).

#### 22.2.6.4. Crystallization Kinetics

A number of methods are available for deriving reaction kinetics constants from DSC thermograms (Wright, 1984). For example, the thermogram obtained during an isothermal DSC experiment at a temperature at which crystallization of a fat occurs can be analyzed in a way similar to that described earlier for the determination of solid fat content, but in this case the evolution of peak area (representing the formation of solid fat crystals) is related to time rather than temperature (Chong, 2001).

Blaurock and Carothers (1990) and Blaurock and Wan (1990) described a simple way, valid for butteroil, of analyzing isothermal DSC data to characterize the kinetics of early crystallization in a supercooled oil. This approach yielded a single crystallization-temperature dependent combined nucleation/crystal growth constant (which they called NG). The temperature dependence of NG could be modeled with the Arrhenius equation.

Wright (1984) has pointed out that most methods of extracting kinetic data from DSC thermograms involve assumptions that should be validated for the system being studied.

#### 22.2.6.5. Purity

DSC can be used to identify the fat components of a mixture of fats by comparing the thermogram of the mixture with thermograms of the individual fats. This approach has been used to detect the adulteration of butterfat with lard (Wright, 1984).

#### 22.2.6.6. Polymorphism

DSC can be used to demonstrate the presence of polymorphism (Mulder and Walstra, 1974). When polymorphism is present, the thermograms for samples of the same fat pre-conditioned thermally in different ways will have different shapes.

A combination of isothermal and heating DSC scans can be used to study polymorphic behavior in some detail, as described above.

The polymorphic behavior of milk fat complicates the interpretation of thermograms obtained for milk fat, milk fat fractions and high-fat dairy products for purposes other than the study of polymorphism itself. The heating or cooling rate used during DSC, as well as the pre-conditioning regime, can affect the behavior of the fat. Wright (1984) has pointed out that careful choice and control of experimental conditions is necessary for reproducibility and interpretability. The results obtained with DSC while heating are largely independent of effects such as supersaturation, supercooling and diffusion, whereas these phenomena occur during cooling (Norris *et al.*, 1971; Wright, 1984). In spite of this, perhaps the simplest approach is to melt the sample completely prior to analysis to erase all “thermal memory” and then carry out a cooling DSC scan. Cooling thermograms have been found to be more reproducible and easier to interpret because during cooling, only exothermic crystallization occurs; heating scans are more complex because polymorphic transformations can occur in addition to endothermic melting (ten Grotenhuis *et al.*, 1999). However, it can be argued that DSC analyses should routinely include both heating and cooling scans (Wright, 1984).

The American Oil Chemists’ Society has published Recommended Practice Cj 1–94 for determining the DSC melting properties of fats and oils. The method is limited in terms of accuracy and reproducibility to the determination of onset temperature and completion of melt (Firestone, 1998).

The theory and principles of DSC, the design and calibration of DSC instruments, and the analysis of experimental data are described by Daniels (1973), Wright (1984), Hatakeyama and Quinn (1994) and Laye (2002).

### 22.2.7. X-Ray Diffraction

X-Ray diffraction (XRD) is the principal means of determining the structures of crystals. It is a technique in which a collimated X-ray beam is directed at a single crystal of the material under investigation or, as is more usual in the study of fat crystals, a sample comprising a large number of randomly orientated crystals. The latter variant, which is the most commonly used generally, is called powder diffraction (Cullity, 1956). Both variants yield essentially the same basic information about structure after data analysis. The following descriptions relate to powder diffraction specifically.

By far the greater proportion of incident X-radiation is transmitted by a crystalline sample. However, a small fraction is scattered (effectively reflected) in all directions by every motif in the material, without change in wavelength. The *motif* is the repeating unit of pattern in a crystal; it is the TAG molecule in the case of a fat crystal. Motifs can be considered to be located at or near the intersections of an imaginary 3-dimensional grid called the *crystal lattice* and the intersections are called *lattice points* (Hammond, 1997).

The scattered X-rays mutually interfere, either destructively or constructively. Almost total destructive interference occurs in all but certain very specific directions in which constructive interference is possible (Sears *et al.*, 1982). Constructive interference in a given direction results in a diffraction beam that is intense relative to the sum of all other rays scattered in the same direction.

The planes of motifs in a crystal thus act essentially as diffraction gratings (Bueche, 1986). Diffraction of electromagnetic radiation by crystals can occur only when the wavelength of the radiation is of the same order of magnitude as the regular repeat distance between motifs. This is the basis of the usefulness of using X-rays to determine crystal structure.

A diffraction beam is produced by constructive interference when the path-length difference between reflections from the motifs in any two parallel planes of motifs in a crystal lattice is equal to a whole number of wavelengths. The angle between the diffraction beam and the lattice planes (the angle of reflection) is equal to the angle between the incident X-ray beam and the lattice planes (the angle of incidence). These phenomena are quantified by the Bragg law (Cullity, 1956):

$$n\lambda = 2d \sin \theta \quad (3)$$

where  $\lambda$  is X-ray wavelength

$d$  is lattice spacing (the distance between two adjacent parallel planes of motifs)

$\theta$  is angle of incidence = angle of reflection.

$2\theta$  is angle of diffraction

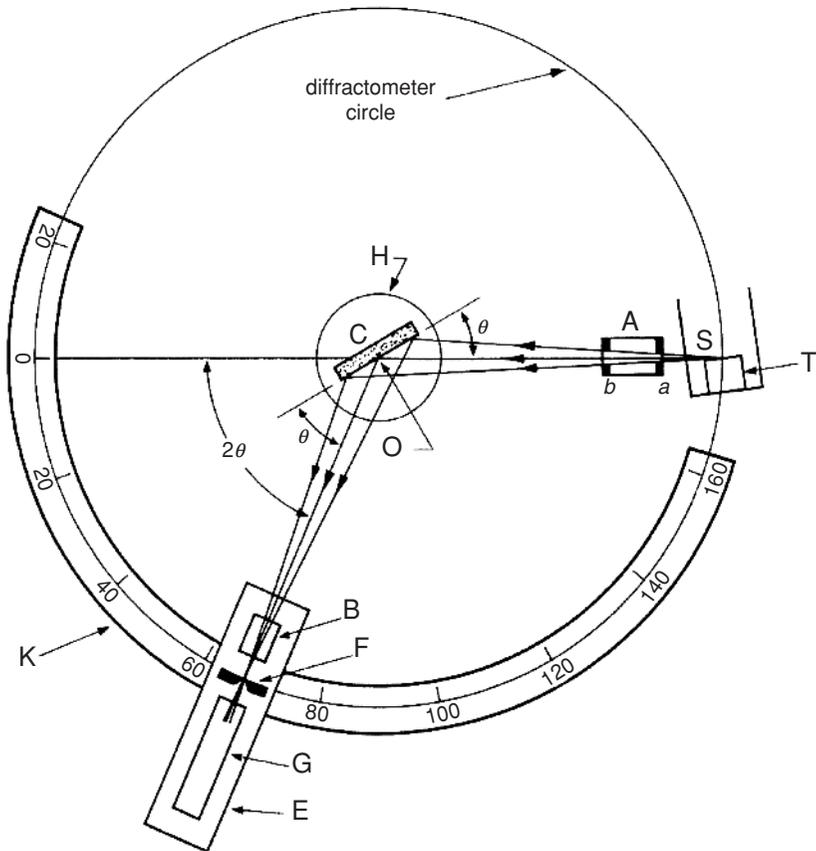
$n$  is an integer representing a whole number of wavelengths.

Conventional X-ray sources produce beams that contain rays of different wavelength. Equation (3) is satisfied by any wavelength for which a set of lattice planes exists in the crystals at an angle  $\theta$  to the incident beam. As a large number of wavelengths can meet this criterion, the diffraction pattern is potentially quite complex (Bueche, 1986).

The structure of a crystal is solved in three steps (Cullity, 1956). Firstly, the size and shape of the unit cell (a crystal lattice consists of identical unit cells) is found from the angular distribution of the diffraction beams. Secondly, the number of molecules per unit cell is computed from the size and shape of the unit cell, the chemical composition of the sample and the sample's measured density. Lastly, the positions of the molecules within the unit cell are deduced from the relative intensities of the diffraction beams. Data analysis, which is complex, is described by Woolfson and Fan (1995) and Clegg (2001).

An X-ray powder diffractometer (Figure 22.3) comprises an X-ray source, the crystalline sample in a sample holder, and a detector for detecting and measuring the intensities of the diffraction beams (Cullity, 1956; Pecharsky and Zavalij, 2003). The incident and diffraction beams are defined and collimated by suitable means. The sample holder, containing a flat powder sample, the detector and the X-ray source are at three points of a triangle. The sample holder can be made to rotate about its axis, and the detector can be made to move around the circumference of a circle (the diffractometer circle) the center of which coincides with the middle of the sample face, but the plane of which is at right angles to this face. The X-ray beam from the source (which is at a fixed point on the same circumference) strikes the face of the powder sample, and the diffraction beam (if the angle is such that one exists) is reflected into the detector. The rotation of the sample holder and the travel of the detector are mechanically coupled. This allows the angle of incidence to be varied while ensuring that the angles of incidence on, and reflection from, the sample are always equal. In some instruments, the sample holder is nonrotating, and the detector and source travel circumferentially in opposite directions to achieve the same result (Cullity, 1956; Pecharsky and Zavalij, 2003).

X-Ray diffraction is carried out using both angles of incidence in the range  $0$ – $3^\circ$  (small angle X-ray diffraction or SAXD) or relatively large angles of incidence (wide angle X-ray diffraction or WAXD). The former are



**Figure 22.3.** Schematic diagram of an X-ray diffractometer. A and B: special slits that define and collimate the incident and diffracted beams; C: powder specimen; E and H: mechanically coupled supports of the detector and powder specimen; F: slit at focal point of diffraction beams; G: detector; K: graduated angle scale; O: diffractometer axis; S: line focal spot on target, T, of the X-ray tube. (Reproduced with permission from Cullity, 1956.)

used for investigating large length-scale crystal structures. Both ranges are used in the study of milk fat polymorphism.

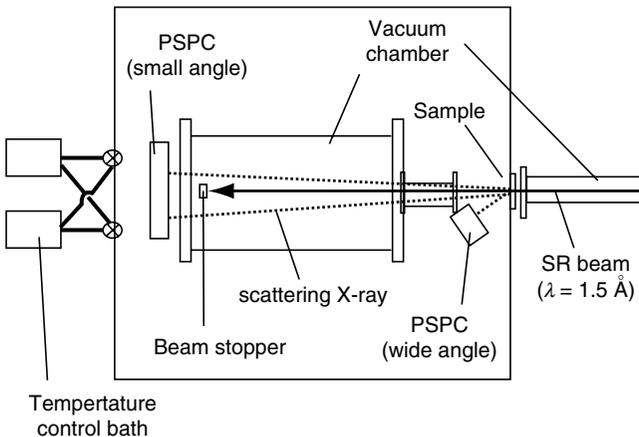
In a diffraction experiment, the angle of incidence is varied (scanned) either step by step or continuously, and the intensities of diffraction beams detected and recorded as a function of angle. Some diffractometers are designed to scan in both SAXD and WAXD modes simultaneously.

The three most commonly used detectors are the gas proportional counter, the scintillation detector and solid-state detectors (Pecharsky and

Zavalij, 2003). Position-sensitive detectors (also called area detectors), based either on a gas-filled ionization chamber or an image intensifier coupled to a video camera detect and record diffraction beam intensity in two dimensions simultaneously, a feature that greatly enhances the speed of data collection (Drenth, 1999).

X-Rays are generated using either conventional X-ray tubes (most commonly) or synchrotrons. In a synchrotron, an extremely large and expensive facility (Drenth, 1999), electrons (or positrons) circulate at an extremely high velocity in a large storage ring (ten to several hundred metres in diameter). The particles emit X-rays owing to their continuous centripetal acceleration towards the center of the ring as their circular direction of travel is maintained by applied magnetic fields. The X-ray beam, of an adjustable and controllable wavelength, can be directed down a straight beam line (tangential to the ring) from the ring to the diffractometer. Synchrotron X-radiation is highly collimated, has extremely high brilliance (a quality related to photon flux) and has a high degree of monochromaticity, among other useful characteristics (Clegg, 2001; Pecharsky and Zavalij, 2003). A synchrotron X-ray diffractometer is shown schematically in Figure 22.4.

There is no essential difference between conventional X-rays and synchrotron X-rays with respect to their use in the investigation of crystal structure. However, the brilliance of synchrotron X-rays (which is currently some ten orders of magnitude greater than that of conventional X-rays), combined with the use of position-sensitive detectors, allows very rapid

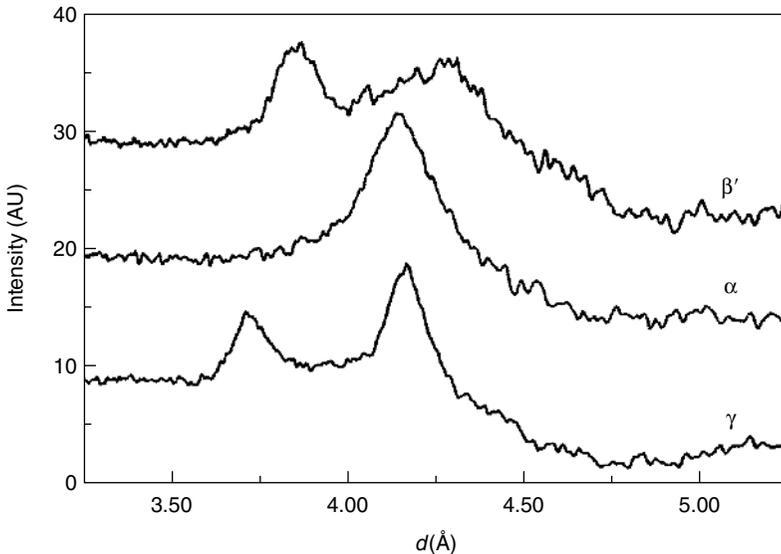


**Figure 22.4.** Schematic diagram of a synchrotron X-ray diffractometer. PSPC: position sensitive proportional counter; SR beam: synchrotron radiation beam. (Reproduced with permission from Ueno *et al.*, 2000.)

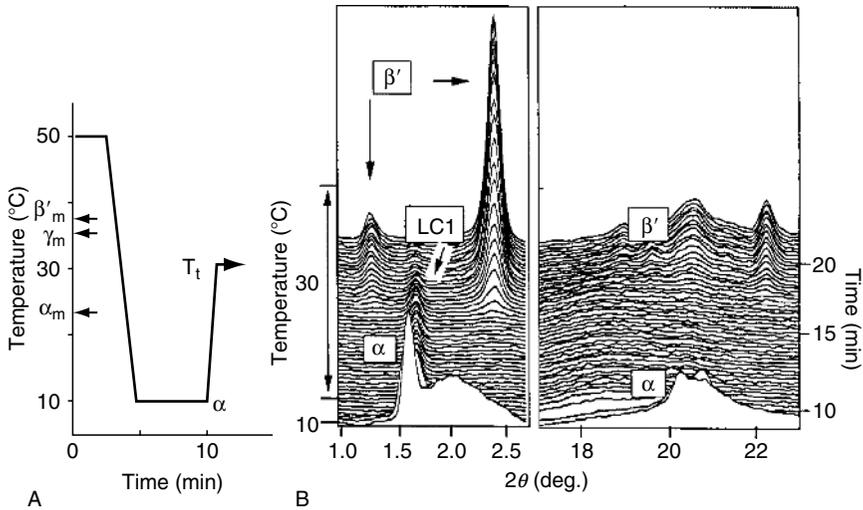
angle scans to be performed, which in turn allows the collection of time-resolved diffraction data. This is extremely useful in studying the dynamics of fat crystallization processes at both constant and time-varying temperature (Sato *et al.*, 1999; Sato, 2001).

The results of a diffraction experiment in which a single angle scan is carried out are presented as a two-dimensional plot of diffraction beam intensity versus angle of diffraction (usually expressed as  $2\theta$ ; Figure 22.5). The results of a time-resolved diffraction experiment are presented as a three-dimensional plot in which the  $x$ -axis is the angle of diffraction, the  $y$ -axis is time (and temperature when temperature is varied with time during the experiment), and the  $z$ -axis is intensity (Figure 22.6). The plot is essentially a non-continuous response surface formed by the intensity versus angle traces of single scans carried out at successive short intervals of time. Examples of such plots for milk fat are presented by, for example, Sato *et al.* (1999) and Sato (2001).

In either case, the plot can be regarded as a “fingerprint” of the crystal structure, and can be used to identify crystals by comparison with stored



**Figure 22.5.** Characteristic two-dimensional X-ray powder diffraction patterns of the three polymorphic forms of milk fat crystals. The pattern of the  $\gamma$ -modification was obtained at  $-50^{\circ}\text{C}$  after cooling at a rate of  $20^{\circ}\text{C}/\text{min}$ . The  $\alpha$ -diffraction pattern was obtained at  $-5^{\circ}\text{C}$  after cooling at a rate of  $5^{\circ}\text{C}/\text{min}$ . The  $\beta'$ -diffraction pattern shown is for milk fat held at  $20^{\circ}\text{C}$  for 30 min. AU = arbitrary units;  $d$  = angle of diffraction. (Reproduced with permission from ten Grotenhuis *et al.*, 1999.)



**Figure 22.6.** (A) Temperature variation profile during, and (B) three-dimensional plots of the results obtained from, a time-resolved synchrotron X-ray diffraction experiment on SOS (1,3-distearoyl-2-oleoyl-*sn*-glycerol) without annealing.  $\alpha$  and  $\beta'$ : polymorphic forms; LC1: liquid crystalline structure 1. (Reproduced with permission from Sato *et al.*, 1999.)

plots for crystal structures that have been solved. This is very useful in the study of milk fat polymorphism.

The principles of X-ray diffractometry are described by Cullity (1956) and Pecharsky and Zavalij (2003).

### 22.2.8. Combined DSC and XRD

DSC and XRD are the methods of choice for studying fat polymorphism and crystalline phase transitions. However, neither is sufficient on its own; they are complementary techniques (Birker and Padley, 1987; ten Grotenhuis *et al.*, 1999). XRD can be used to measure the amounts of different polymorphs in a mixture, as the intensities of the diffraction beams due to a constituent of a mixture depend on the proportion of that constituent in the mixture (Cullity, 1956). Changes in the intensities and angular positions of diffraction beams are used as a measure of polymorphic changes. However, XRD is not sensitive to transitions within the same polymorphic form; changes in molecular composition and crystal size, and thermal shrinkage or expansion effects, tend to be confounded (ten Grotenhuis *et al.*, 1999).

DSC, in contrast, is sensitive both to phase transitions within polymorphs and to polymorphic transformations, and the equipment needed is

less elaborate than that required for XRD (ten Grotenhuis *et al.*, 1999). However, DSC cannot identify crystal forms unequivocally (Birker and Padley, 1987), and when several transitions occur simultaneously it can be difficult to quantify their separate effects. Further, in the case of fats, interpretation of data is complicated by the dependence of fat polymorph heat of transition on TAG composition (ten Grotenhuis *et al.*, 1999).

The misinterpretation or underutilization of data obtained using either XRD or DSC alone can be overcome by using both techniques in a given investigation (ten Grotenhuis, 1999).

### 22.2.9. Coupled DSC and XRD

Even when DSC and XRD are both used, quite separate instruments are involved. This leads to difficulties in reconciling results owing to differences in sample thermal history/conditioning, sample dimensions and sample temperature control and uniformity. These difficulties can be entirely overcome by coupling XRD and DSC together in the same instrument and making both types of measurement simultaneously on the same sample.

A custom-made instrument in which high sensitivity DSC and time-resolved synchrotron SAXD and WAXD can be performed simultaneously on the same sample has been built by a French research group (Keller *et al.*, 1998). This instrument is claimed to be an advance over earlier attempts to couple DSC and XRD. The single sample is held in a thin glass capillary tube, and all experimental data are collected by a single computer. Local inhomogeneity in sample temperature caused by absorption of X-ray energy has been shown not to perturb appreciably the measurement of true sample temperature. Details of the design and attributes of the instrument are given by Keller *et al.* (1998). This instrument has been used extensively to study phase change behavior in anhydrous milk fat and cream (Lopez, *et al.*, 2000, 2001a, b, c, d, e, 2002, 2005). This work showed, *inter alia*, that the brilliance of synchrotron X-radiation, and the coupling of both SAXD and WAXD with DSC are essential for interpreting the complex thermal and structural behavior of fat in dispersed systems such as cream.

Coupling of DSC and XRD has considerable potential for improving the control of polymorphism in the processing of fat-based foods (Ollivon *et al.*, 2001; Allais *et al.*, 2003).

### 22.2.10. Cooling Curves

The cooling curve of a fat is a plot of temperature versus time obtained by measuring the temperature of a sample of the molten fat while the sample is

cooled under standard conditions. The cooling curve is a measure of speed of crystallization, or rate of polymorphic change, and is useful in assessing or interpreting the behavior of a fat or fat-based product in food manufacture. It is a dynamic measure of phase change behavior, and complements solid fat index as measured by dilatometry, or solid fat content as measured using NMR; these are static or equilibrium measures of phase change behavior (Rossell, 1986). The cooling curve is particularly useful in chocolate manufacture (Padley *et al.*, 1972; Rossell, 1986).

In the International Union of Pure and Applied Chemistry (IUPAC) standard method 2.132 (Paquot and Hautfenne, 1987), the cooling curve of the molten sample is obtained by placing the vacuum jacketed flask containing the sample in an ice-water bath and observing sample temperature every minute. The cooling curve is compared with the cooling curve for a sample of soybean oil (which does not crystallize under the conditions of the test) obtained under identical conditions. The important features of the curve are the “prime stay temperature” (the temperature at which the sample and soybean oil curves start to diverge), the temperature minimum (at which the rate of release of latent heat of crystallization equals the rate of heat loss by cooling) and the subsequent temperature maximum (caused by the rate of release of latent heat exceeding, for a time, the rate of heat loss by cooling). The times at which these temperatures are reached, and the general shape of the curve, are also useful pieces of information.

The British Standard method BS 684: Section 1.13: 1976 (BSI, 1976d) is similar. However, the sample is deliberately stirred during the measurement in such a way that small fat crystals formed in the upper parts of the apparatus are carried down into the sample, where they act as crystallization nuclei. This causes crystallization to occur in the most stable polymorphic form, making the test more suitable than the IUPAC one for assessing the performance of fat in chocolate manufacture (Rossell, 1986).

## 22.2.11. Optical Methods

### 22.2.11.1. Light and Polarized Light Microscopy

The rate of growth of fat crystals can be measured by photographing growing crystals at regular intervals using a light microscope equipped with a camera and a length-graduated ocular, followed by suitable analysis of crystal size measurements (Kawamura, 1979).

van Putte and Bakker (1987) described a system for measuring nucleation rate during fat crystallization in which the number of crystals per unit volume was counted subjectively as a function of time using a light

microscope equipped with a Fuchs–Rosenthal counting chamber (commonly used for counting blood cells).

The use in microscopy of plane-polarized light (in which the wave motion of the light occurs in one plane only, perpendicular to the direction of travel) is particularly useful for observing and imaging fat crystals. A polarized light transmission microscope comprises a light source, an optical polarizer (a Nichols prism), a stage on which can be placed a small fat sample held between a temperature-controlled glass slide and a coverslip, an optical analyzer (another Nichols prism) and a means of image capture (Slayter and Slayter, 1992). The polarizer and the analyzer are in the “crossed Nichols” orientation (i.e., at  $90^\circ$  to one another).

Completely molten fat is optically isotropic (i.e., its refractive index for plane-polarized light is the same in all directions). When the sample on the microscope’s stage is molten, the crossed Nichols prisms prevent any polarized light leaving the analyzer, and no image can be detected. Fat crystals, on the other hand, are optically anisotropic, typically birefringent (having two principal refractive indices) (Slayter and Slayter, 1992; Narine and Marangoni, 1999). A birefringent material present between polarizer and analyzer splits the plane-polarized light into two rays, called the ordinary ray and the extraordinary ray. Constructive interference of these rays in the analyzer results in light passing through the analyzer (James and Tanke 1991; Slayter and Slayter, 1992). Thus, as soon as crystallization begins, an image is detectable.

Herrera *et al.* (1999a) measured the change of fat crystal size distribution with time during crystallization using a polarized light microscope equipped with a still camera, by means of which photographs were taken at regular intervals. The photographs were scanned, and the size distribution at each time, based on longest crystal dimension, was determined using suitable software.

The coupling of polarized light transmission microscopy with image analysis has proved a useful way of measuring the rate of crystallization. For example, Wright *et al.* (2001a) described a system in which a video camera was used to capture the image of the crystallizing fat sample produced by the microscope, at intervals of 15 s. The images were processed using suitable software. After “thresholding,” subtracting an initial image from every subsequent image, the numbers of black and white pixels in each image were determined. The number of black pixels, representing crystal mass, was determined as a function of time to give the crystallization rate. Thresholding is critical, as it must be done in such a way that all of the solid mass is represented in the resulting thresholded image (Narine and Marangoni, 1999). Video microscopy is described in detail by Sluder and Wolf (1998).

Other methods of imaging fat crystals and fat crystal networks (not all of them optical) include confocal laser scanning fluorescence microscopy, multiple photon microscopy, atomic force microscopy and electron microscopy (Narine and Marangoni, 1999).

#### 22.2.11.2. Laser Light Diffraction Spectrophotometry

This technique can be used to determine the crystal size distribution in an isothermally crystallizing oil (van Putte and Bakker, 1987). Laser light is caused to shine through and onto a sample of the crystallizing mixture by inserting the mixture into the paths of laser beams, and the angles and intensities of light diffracted by the crystals are measured by means of a lens system and light-sensitive detectors. The angle of diffraction is related to particle size, and the intensity is related to the proportion of any particular size present. Data analysis is complex.

The growth rate of fat crystals can be found by measuring the particle size distribution as a function of time, and calculating growth rate as the rate of increase of maximum crystal size (van Putte and Bakker, 1987).

Visible light diffraction is the same phenomenon as X-ray diffraction.

#### 22.2.11.3. Visible Light Absorption Spectrophotometry

The development of turbidity with time during isothermal crystallization of an oil, which is the result of crystal formation, can be used to study nucleation and crystal growth (e.g., Herrera *et al.*, 1999b; Dibildox-Alvarado and Toro-Vasquez, 1997; Wright *et al.*, 2000, 2001a). The sample is temperature-conditioned to erase thermal memory and cooled to crystallization temperature in a temperature-controlled cell or cuvette in a spectrophotometer. The absorbance or transmittance (optical density) of the sample with respect to visible light is then measured as a function of time. The induction or nucleation time is taken as the time from the start of isothermal holding to the time at which the absorbance begins to increase or the transmittance to decrease.

Herrera (1994) described a sophisticated version of this technique in which polarized laser light was used in much the same way as in polarized light transmission microscopy. The laser light beam passed from the source (a helium–neon laser) to a photosensor (a cadmium–sulphide photodiode) through, in sequence, an optical polarizer, the crystallizing fat sample in a temperature-controlled glass cell, and an optical analyzer in the crossed Nichols position relative to the polarizer. As soon as crystallization began, the optically anisotropic fat crystals that then appeared resulted in light

passing through the analyzer to be detected by the photosensor. The induction time was taken as the interval between the instant at which the sample reached the isothermal crystallization temperature and the time at which light was first detected.

Similar systems, in which the experimental set-ups were conveniently in the form of polarized light transmission microscopes, have been described by Koyano *et al.* (1989) and Ng (1989).

Absorption spectrophotometry is a satisfactory way of measuring induction time, but is quite unsuitable for the kinetic characterization of subsequent crystallization, except in the early stages; minimum transmittance does not correspond to the end of crystallization, transmittance is proportional to the extent of crystallization only when no multiple scattering of the incident light occurs, and an observed decrease in transmittance can be due to light refraction (Marangoni, 1998).

#### 22.2.11.4. Visible Light Reflectometry

Wright *et al.* (2001a) described a system for continuously monitoring crystallization in which a beam of visible light was directed onto the crystallizing sample perpendicularly from above. A lens system and a matrix of light detectors were located perpendicularly above the sample. The solid-liquid (crystal-oil) boundaries in the sample scattered the incident light beam, and the intensity of the scattered light was measured by the detectors. The output signal, proportional to crystal mass, was recorded as a function of time.

### 22.3. Thermal Properties: Critical Temperatures

When a fat or oil is heated, thermal instability may cause decomposition, and depending on the temperature reached, subsequent combustion of volatile gaseous decomposition products (Mehlenbacher, 1960). The thermal stability of fats and oils is thus essentially a chemical characteristic. However, stability is characterized by measuring certain critical temperatures, the *smoke*, *flash* and *fire* points, at which certain heat-induced changes become apparent. It is appropriate, therefore, to include here methods for measuring these critical points.

Measurement of smoke, flash and fire points is carried out subjectively by observing the surface of an oil sample while the sample is being heated. (The critical temperatures are higher than the upper limit of the melting point range of a fat.) The smoke point is the temperature at which the sample begins to give off a continuous stream of bluish smoke, observable

by means of a light beam directed across the surface of the sample. The flash point is the lowest temperature at which a flash of flame appears at any point on the sample surface when a flame is applied near the surface. The fire point is the lowest temperature at which combustion continues for at least 5 s when a flame is applied (Rossell, 1986).

Standard methods for the determination of the critical temperatures are published by the AOCS (AOCS Official Methods Cc 9a–48 (smoke, flash and fire points by an open cup method), and Cc 9b–55 and Cc 9c–95 (flash point by open cup methods), Firestone, 1998) and by the British Standards Institution (BS 684: Section 1.8: 1976 (smoke point), BSI, 1976b; and BS 684-1.17:1998/ISO 15267:1998 (flashpoint by a closed cup method), BSI, 1998b).

## 22.4. Rheological Properties

### 22.4.1. Introduction

Rheology is usually defined as *the study of the deformation and flow of matter* (Barnes *et al.*, 1989). Rheology is used extensively in investigating and characterizing the nature and microstructure of milk fat and fat-based dairy products. Rheological measurements are useful also in objectively measuring properties related to texture, in measuring functionality, in providing data for process modeling and control, and in quality control.

In the following, rheological behavior is defined, a practical distinction is made between *solids* and *liquids*, and the requirements for the rheological characterization of materials are stated. Then, common *rheometers* (rheological instruments with well defined geometries) and their use in the measurement of fundamental rheological properties are described. Lastly, applications of rheometers, and applications of empirical and imitative rheological instruments and techniques to specific milk fat-based products (milk fat and milk fat fractions, butter, cheese, cream, ice cream, and chocolate) are described. A very wide range of instruments and experimental methods has been used in the rheological study of these products. *Fundamental*, *empirical* and *imitative* rheological techniques and measurements, and the distinctions between them, are defined in Section 22.4.3.

### 22.4.2. Rheological Behavior and Material Classification

All real materials fall rheologically between two extremes: the perfectly elastic Hookean solid, for which stress is directly proportional to strain, and the Newtonian liquid, for which (shear) stress is directly proportional to (shear) strain rate. Strain can be defined as deformation relative to a reference length, area or volume (Barnes *et al.*, 1989); it is dimensionless. Strain

rate (reciprocal time) is the rate of change of strain with time. A stress (force per unit area) must be applied to a material to cause a strain, or is set up in a material by the imposition of a strain.

Real materials are neither truly Hookean nor truly Newtonian, though some exhibit Hookean or Newtonian behavior under certain conditions (Barnes *et al.*, 1989). Real materials may exhibit nonlinearity, which is a lack of direct proportionality between stress and strain, or between stress and strain rate. Real materials may exhibit either predominantly elastic behavior or predominantly viscous behavior, or a measurable combination of the two, depending on the stress or strain and the duration of its application (Barnes *et al.*, 1989). Such materials are termed viscoelastic. Barnes *et al.* (1989) pointed out that it is better to classify *rheological behavior* than to classify *materials*; a given material can then be included in more than one rheological class depending on experimental conditions.

However, for practical purposes, it is useful to distinguish between solids and liquids according to the following definitions (Barnes *et al.*, 1989):

- A *solid* is a material that will not continuously change in shape when subjected to a given stress.
- A *liquid* is a material that will continuously change in shape (i.e., will flow) when subjected to a given stress, no matter how small that stress is.

A given material may be a solid under some conditions and a liquid under others; for example a plastic material is a solid at stresses lower than its yield stress but a liquid at higher stresses. The term *semi-solid* is a convenient, though imprecise, description of materials that when unsupported by a container, change shape (deform) under the influence of gravity under ambient conditions. Many fat-based foods are semi-solid.

Viscoelastic materials can be divided into viscoelastic solids and viscoelastic, or simply elastic, liquids. All viscoelastic liquids are non-Newtonian, but not all non-Newtonian liquids are viscoelastic. Non-Newtonian liquids show nonlinear rheological behavior, and this may be time dependent (Barnes *et al.*, 1989).

### 22.4.3. Rheological Characterization of Materials

The fundamental rheological characterization of a material requires the experimental determination of a constitutive equation (a rheological equation of state) that mathematically relates stress and strain, or stress and strain rate. The constants in the constitutive equation are the rheological properties of the material.

The necessary experimental data must be obtained using rheometers, instruments in which measurements can be made that provide values of

stress, and either strain or strain rate, that are related only by the rheological behavior of the sample (i.e., are independent of sample shape and size, and instrument geometry). Suitable treatment of these data leads to the constitutive equation that describes the material's rheological behavior in the ranges of the variables investigated.

Where the complexity of the instrument geometry and/or the sample geometry prohibits analysis of stress-strain conditions during testing, the test and results obtained are *empirical* only; the data cannot be converted into values of fundamental properties. However, such data can still be very useful. Sectilometry, the measurement of the force required to cut with a taut wire, used in characterizing butter, is an example of an empirical approach to measuring rheological behavior, in this case in terms of "spreadability"; while not a fundamental method, it is simple, fast and inexpensive, and gives useful (if rheologically non-fundamental) information.

An *imitative* test is a type of empirical test in which the test geometry and test conditions are designed to mimic those that a product experiences in end use. Such tests can provide very useful means of assessing the rheological functionality of fat-based products. A good example of an imitative test is one for Mozzarella cheese stretchability in which the two halves of a divided pizza base covered with the cheese can be moved apart after cooking, and the extent of stretching to fracture measured (Guinee *et al.*, 1999). Imitative tests that simulate jaw action are useful for measuring product attributes related to texture; texture itself can be defined as the human physiological-psychological perception of a number of rheological and other properties of a food and their interactions, and thus cannot itself be measured by wholly objective means (McCarthy, 1987).

Rheometers can be divided into two broad types: *viscometers*, used to measure the rheological properties of liquids, and *solids rheometers*, used to measure the rheological properties of solids. Viscometers and solids rheometers are not mutually exclusive in application; some viscometer geometries can be used for testing solids, while some solids rheometer geometries can be used for testing (viscous) liquids.

Rheometrical data may be fundamental or empirical in nature depending on the conditions of measurement relative to the sample's rheological behavior; conditions must be carefully controlled when fundamental data are required.

#### **22.4.4. Viscometers and the Measurement of Fundamental Viscous Properties**

A viscometer is an instrument in which shear stress and shear rate (the strain rate in shear) can be measured at the same location in the instrument independently of the properties of the material being tested.

Viscometers can be divided into rotational instruments and axial flow instruments. Rotational instruments include concentric cylinder (cup and bob), cone and plate and parallel disc viscometers, while axial flow instruments include capillary, slit and extrusion rheometers.

In rotational instruments, one member (e.g., the cup in a concentric cylinder viscometer) rotates while the other (e.g., the bob) remains stationary. The sample is held, and sheared, in the gap between the two. In a controlled shear rate measurement, the rotational speed is constant, and the torque on one member caused by the viscous resistance to flow exerted by the sample is measured. In a controlled stress measurement, a constant torque is applied to one member and its speed of rotation measured. Controlled stress instruments are particularly useful for measuring yield stress, the minimum stress causing flow of a plastic material.

In general, shear stress at one location (e.g., the bob surface in a concentric cylinder viscometer) is calculated from the dimensions of the sample gap and the measured or applied torque. Shear rate is calculated at the same location from sample gap dimensions and rotational speed. By making experimental measurements over a range of speeds or torques, the flow curve (shear stress versus shear rate) of the sample can be established. Suitable mathematical treatment of the flow curve data yields the sample's constitutive equation and rheological properties.

In a wide-gap concentric cylinder instrument, the shear rate at a given location depends on the rheological behavior of the sample. This complicates, but does not prohibit, evaluation of shear rate. In a narrow-gap concentric cylinder instrument (bob radius/cup radius  $>0.97$ ), the shear rate may be considered constant at the average value in the gap; shear rate then depends only on radii and rotational speed, making its evaluation easy (Barnes *et al.*, 1989).

The software of most commercial concentric cylinder viscometers calculates only an average shear rate, even when the gap is wide according to the above criterion.

Cone and plate instruments have the advantage that the shear rate can be considered constant at all points in the gap at a given rotational speed provided the cone angle is less than  $4^\circ$ . They have the disadvantage of being unsuitable for testing materials containing particles, because the gap between cone and plate approaches zero at the cone tip.

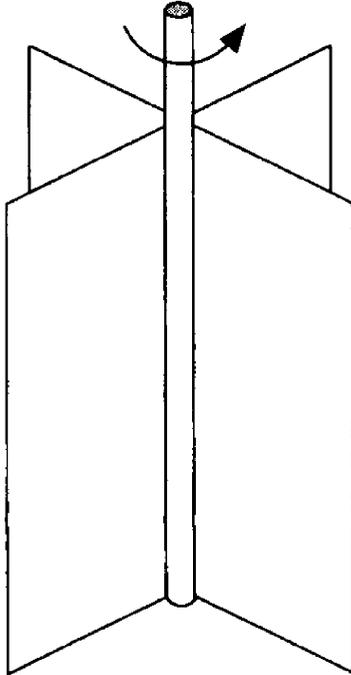
The parallel disc instrument does not have this limitation, but shear rate is a function of radius, complicating data analysis.

A variation of the concentric cylinder viscometer is the rotating cylinder in an "infinite sample". In this controlled (low) shear rate instrument, the sample is contained in a vessel of such large diameter relative to the cylinder's diameter that the vessel wall exerts no influence on the shear

caused by the cylinder's rotation. The torque required to rotate the cylinder at various controlled speeds is measured (Skelland, 1967).

The vane viscometer is yet another form of the concentric cylinder instrument, in which the bob is replaced by a rotor with four blades or vanes each attached by one edge to a vertical shaft, at  $90^\circ$  intervals around the shaft (Figure 22.7). This geometry, which can be used either with a cup or in the "infinite sample" mode, is particularly useful for measuring yield stress, and can also be used to measure the rheological properties of non-Newtonian liquids. Its advantages are described by Gunasekaran and Ak (2002).

In axial-flow viscometers, the sample is made to flow through a duct of regular cross-section. Capillary (circular cross-section) and slit (rectangular cross-section) viscometers are controlled stress instruments: a known pressure difference (which causes shear stress in the sample) is applied over the duct length, and the resulting volumetric flow rate measured. In the extrusion viscometer, a controlled shear rate instrument, the sample is extruded through a capillary tube by the action of a constant speed piston, acting on the sample in a cylindrical reservoir to which the capillary is attached. The pressure difference between the ends of the capillary is measured.



**Figure 22.7.** The rotor of a vane rotational viscometer. (Reproduced with permission from Gunasekaran and Ak, 2002.)

In both types of axial flow instrument, the shear stress at the duct wall is calculated from the duct dimensions and the pressure drop along the duct length, while shear rate at the same location is calculated from duct dimensions and volumetric flow rate. The wall shear rate depends on the rheological properties of the sample, complicating but not prohibiting its evaluation.

In viscometry, it is usually necessary to correct for end effects (e.g., entrance and exit effects in axial flow instruments) and for slip between sample and viscometer surfaces.

Most current commercial viscometers worthy of the description *rheometer* are of the rotational type, and many are sophisticated and versatile instruments. Axial flow instruments are often user-designed and built.

Viscometers of relatively complex geometry, for example the Ostwald glass U-tube viscometer, can be used to measure the viscosity of Newtonian liquids, which is independent of shear rate and time, after calibration with a Newtonian liquid of known viscosity. Such instruments cannot be used for rheologically characterizing non-Newtonian liquids, and therefore cannot be classed as rheometers, as geometrical complexity prevents evaluation of shear stress and shear rate at a given location independently of sample rheological behavior.

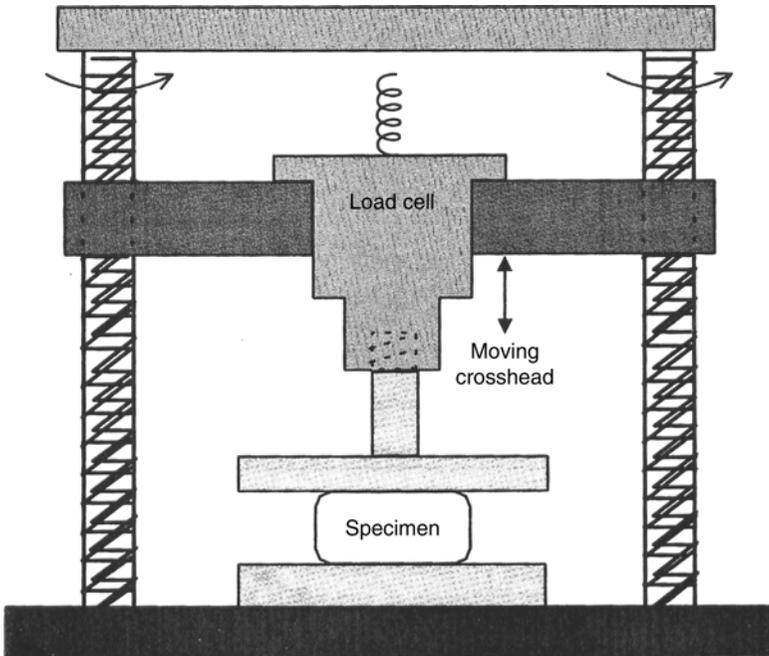
The principles of rotational and axial flow viscometers, and methods of mathematically manipulating measured experimental data, are described by Van Wazer *et al.* (1963), Skelland (1967), Barnes *et al.* (1989) and Steffe (1992).

#### **22.4.5. Solids Rheometers and the Measurement of Fundamental Elastic Properties**

Solids rheometers are instruments in which solid samples of regular shape are subjected to well-defined deformations, and the forces required to do this are measured.

The most common solids rheometers are of the “universal testing machine” (UTM) type (Gunasekaran and Ak, 2002). Such an instrument comprises a horizontal stationary base-plate and a crosshead, above the base-plate, that can move up or down vertically at a variable constant speed (Figure 22.8). The crosshead incorporates a load cell for measuring and recording force. The crosshead speed, position and direction of movement relative to the base-plate are accurately controlled and recorded.

The sample, usually in the form of a cylinder, can be subjected to uniaxial compression (the simplest and most common test), uniaxial tension, shear, bending or torsion. In compression, the sample rests on the base-plate and is compressed by a horizontal flat plate attached to the crosshead when



**Figure 22.8.** Schematic diagram of a universal testing machine (UTM)-type solids rheometer. (Reproduced with permission from Gunasekaran and Ak, 2002.)

the crosshead is made to move downwards. For a tension test, the sample, usually in the form of a dumbbell of circular or thin rectangular cross-section, is attached to both the base-plate and the crosshead by suitable means, and the crosshead is made to move upwards. For shear and bending tests, suitable assemblies for holding and deforming the sample (which is commonly in the form of a cylinder or a right parallelepiped) are attached to the base-plate and crosshead. In the case of torsion testing, the linear motion of the crosshead can be converted to a torque-induced rotation of one end of the sample (which is usually capstan-shaped) by means of a line and pulley system; the sample is twisted about its longitudinal axis.

Paradoxically, diametric compression, in which a disc-shaped sample is compressed diametrically between the crosshead plate and the base-plate, can be used to measure tensile strength (Gunasekaran and Ak, 2002). It is a simpler option than tensile testing because of the difficulty in the latter of satisfactorily gripping food material samples, which are relatively weak.

A double compression test, in which the same sample is subjected to two consecutive compressions and “unloadings”, forms the basis of the Texture Profile Analysis (TPA) technique, which yields mainly empirical

rheological data closely related to texture. The test is fully described by Bourne (2002).

Semi-solid foods, such as soft butter and some cheeses, cannot be formed into samples capable of supporting their own weight. For such foods, "compression" testing takes the form of cone or die penetrometry, in which a cone, die, needle or sphere is made to penetrate the sample (held in a suitable container) either under constant load or at constant speed, and the penetration depth measured as a function of time. Standard methods for penetrometry of fats are published by the AOCS (AOCS Official Method Cc 16-60, Firestone, 1998) and the British Standards Institution (BS 684: Section 1.11: 1976, BSI, 1976c).

Modern solids rheometers are fully computerized. The test parameters (crosshead speed, direction of travel, rate of collection of force-distance data pairs, etc) can be set up on the instrument's computer. The computer then initiates and controls the mechanical action of the instrument and records the measured data.

The force-distance (force-deformation) data can be converted into stress-strain data using the initial sample dimensions and any relevant instrument factors. The stress-strain data can then be mathematically analyzed to yield values of rheological properties such as elastic modulus, tangent modulus, secant modulus, Poisson's ratio, limit of linearity between stress and strain, and resilience (the area beneath the stress-strain curve up to the limit of proportionality) (Gunasekaran and Ak, 2002). These properties can be regarded as quasi-fundamental for real materials provided strains are within the limit of proportionality, and strain rates during testing are relatively high, so that the elastic character of the sample predominates (McCarthy, 1987). Procedures where these conditions exist may be called *small deformation tests* or *fundamental tests*; they measure well-defined rheological properties, inherent properties of the sample independent of instrument geometry, sample geometry, sample size and stress-strain conditions (McCarthy, 1987).

*Large deformation tests*, in which a solid sample is strained to well beyond its linear limit, and often to fracture, are designed to obtain a quantitative measure of a product's functionality in end use. Many large deformation tests are empirical or imitative, and do not yield fundamental rheological or fracture data. However, such tests can, with some materials, be set up and performed in such a way that fundamental information is obtained (McCarthy, 1987).

Fracture properties arguably are not rheological properties, but mechanical properties, given the standard definition of rheology (see above). However, failure of structure in foods, especially high-fat foods, usually begins at strains below the actual fracture point, the point at which the

sample begins to break apart into two or more pieces; it is therefore convenient, if not entirely correct, to include the measurement of fracture properties (e.g., failure stress), in a general discussion of rheological methods.

Details of solids rheometer design, operation and data analysis can be found in Whorlow (1992), Collyer and Clegg (1998) and Gunasekaran and Ak (2002).

#### **22.4.6. Measurement of Linear Viscoelastic Properties**

Linear viscoelasticity is the simplest type of viscoelastic behavior, in which viscoelastic properties are independent of the magnitude of applied stress or strain (Barnes *et al.*, 1989; Gunasekaran and Ak, 2002). Linear viscoelasticity is usually exhibited by food materials at very small strains (Rao, 1992) that cause negligible damage to the food's structure; the phenomenon must therefore be investigated experimentally using small deformation test methods.

Linear viscoelastic properties can be measured in two ways: by static methods or by dynamic methods (Barnes *et al.*, 1989).

In static tests, the sample is subjected to a step change in stress with the resulting strain being measured as a function of time (creep tests), or a step change in strain with the resulting stress being measured as a function of time (stress relaxation tests). Static tests on solid-like materials, in compression, tension, shear or torsion, can be carried out using solids rheometers of the types described earlier, while for liquid-like materials, static tests can be carried out in suitably designed concentric cylinder, cone and plate or parallel disc viscometers. The parallel disc geometry can be used also for carrying out static tests on solid samples. The sophisticated mechanics, electronics and operating software of many modern viscometers and solids rheometers make creep and stress relaxation experiments easy to perform.

The most common dynamic method is oscillatory testing, in which the sample is subjected to a sinusoidal oscillatory strain, and the resulting oscillatory stress measured. The more sophisticated rotational viscometers have the additional capability of dynamically testing liquid-like materials using small angle oscillatory shear. A parallel disc viscometer can be set up for testing solid-like materials (e.g., butter), in oscillatory shear. Some UTM-type solids rheometers, in which the moving crosshead can be made to reciprocate sinusoidally, can be used to test solid-like materials in oscillatory deformation in compression, tension or shear.

A number of highly sophisticated commercial rheometers, rather different in design from the traditional UTM, are now available in which dynamic and static tests on solids in compression, tension, shear and bending can all be carried out using the same instrument.

The four variables in dynamic oscillatory tests are strain amplitude (or stress amplitude in the case of controlled stress dynamic rheometers), frequency, temperature and time (Gunasekaran and Ak, 2002). Dynamic oscillatory tests can thus take the form of a strain (or stress) amplitude sweep (frequency and temperature held constant), a frequency sweep (strain or stress amplitude and temperature held constant), a temperature sweep (strain or stress amplitude and frequency held constant), or a time sweep (strain or stress amplitude, temperature and frequency held constant). A strain or stress amplitude sweep is normally carried out first to determine the limit of linear viscoelastic behavior. In processing data from both static and dynamic tests it is always necessary to check that measurements were made in the linear region. This is done by calculating viscoelastic properties from the experimental data and determining whether or not they are independent of the magnitude of applied stresses and strains.

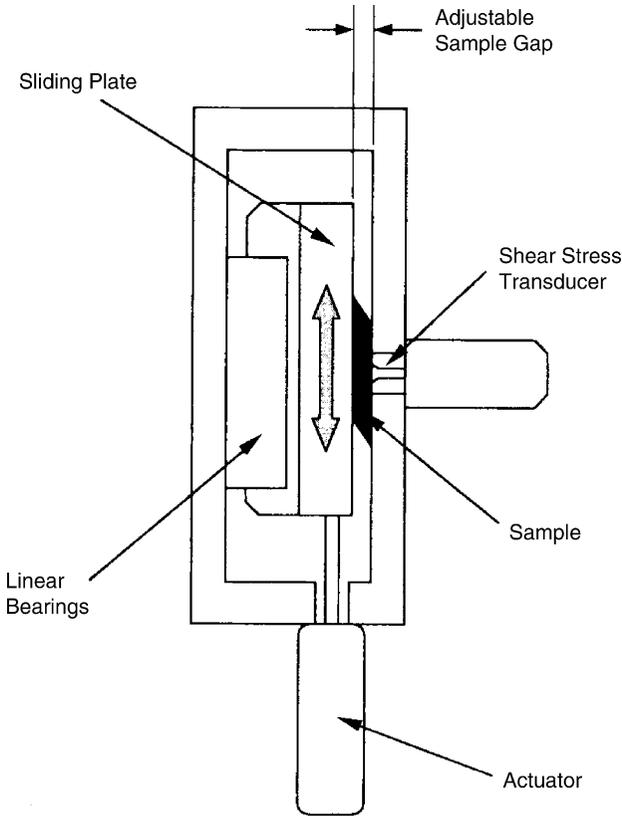
The derivation of fundamental linear viscoelastic properties from experimental data obtained in static and dynamic tests, and the relationships between these properties, are described by Barnes *et al.* (1989), Gunasekaran and Ak (2002) and Rao (1992). In the linear viscoelastic region, the moduli and viscosity coefficients from creep, stress relaxation and dynamic tests are interconvertible mathematically, and independent of the imposed stress or strain (Harnett, 1989).

#### **22.4.7. Measurement of Nonlinear Viscoelastic Properties**

Measurement of linear viscoelastic properties is a useful way of gaining information about a food's microstructure and how this influences the food's rheological character (Narine and Marangoni, 1999; Gunasekaran and Ak, 2002). However, many processing operations, and mastication, involve large, rapid deformations during which viscoelastic behavior is nonlinear.

Although attempts to measure and interpret nonlinear behavior are potentially useful, there are few reports in the literature on the measurement of the nonlinear viscoelastic properties of foods. This has been due to a lack of both suitable instrumentation and suitably developed theory; nonlinear behavior, the predominant form of which is the exhibition of normal stresses, and a dependence of viscosity on shear rate, is much more complex than linear behavior (Gunasekaran and Ak, 2002).

Conventional rheometer geometries such as concentric cylinders, cone and plate and parallel discs are unsuitable, even when the rheometer is designed to allow measurement of normal forces. Many of the disadvantages of such geometries are overcome in the sliding-plate viscometer (Gunasekaran and Ak, 2002). In this instrument (Figure 22.9), the sample (the exact shape and size of which need not be known) is held between a



**Figure 22.9.** Schematic diagram of a true shear sliding-plate rheometer. (Reproduced with permission from Gunasekaran and Ak, 2002.)

stationary flat plate and a second, parallel, flat plate that is oscillated in its own plane at controlled amplitude and frequency. The sample is subjected to large, uniform deformation in true shear, at high shear rates. Shear stress is measured by means of a force transducer flush mounted in the stationary plate (Gunasekaran and Ak, 2002). Gunasekaran and Ak (2002) described ways of analyzing the data obtained with this instrument, specifically in relation to determining the nonlinear viscoelastic properties of cheese.

#### 22.4.8. Measurement of Extensional Viscosity

The extensional viscosity of semi-solid fat-based products such as butter, ice cream and some cheeses can be measured by lubricated squeezing flow rheometry (Campanella and Peleg, 2002; Gunasekaran and Ak, 2002).

In the most suitable test configuration for foods, the sample initially completely fills the gap between two coaxial horizontal discs, one attached to the base and the other to the crosshead, of a UTM-type solids rheometer. Lubrication between the sample and disc surfaces is achieved by Teflon coating the surfaces and/or deliberately lubricating the surfaces with a suitable liquid of very low viscosity.

The sample is subjected to compression by moving the crosshead downwards at a constant speed. The sample is extruded from between the two discs, undergoing elongational or biaxial flow; the sample is stretched radially and azimuthally as it flows outwards between the approaching discs. Lubrication ensures that shear flow cannot occur. Elongational viscosity is calculated directly from the measured force–distance data, disc radius and crosshead speed; no rheological model is required (Campanella and Peleg, 2002).

Lubricated squeezing flow rheometry (and unlubricated squeezing flow rheometry, in which friction between the sample and discs results in radial shear flow) can be used also to measure Newtonian viscosity and the flow properties of non-Newtonian liquids (Campanella and Peleg, 2002).

Lubricated squeezing flow rheometry has two significant advantages over more conventional viscometry: slip between sample and instrument surfaces is an advantage rather than a nuisance, and the sample can be mounted in the instrument with minimal damage to its structure. Damage can be avoided altogether by using the so-called imperfect squeezing flow technique. In this, the sample is allowed to form (develop its structure) in a shallow circular container, or is collected in the container from, for example, a filling machine. The sample is then compressed in its container by a vertically moving disc, which is concentric with the container but of smaller diameter. Artifacts are minimized by making disc diameter, and the gap between disc and container wall, as large as is practicable, and compressing the sample to a very small final height (Campanella and Peleg, 2002).

The theory of squeezing flow rheometry assumes that the sample is nonelastic. Tests on viscoelastic samples should therefore be carried out at low strain rates, to minimize elastic response, and results should be reported as *apparent* elongational viscosity.

Good descriptions of the principal ways of carrying out fundamental rheological measurements of all kinds can be found in Collyer and Clegg (1998) and Whorlow (1992).

## **22.4.9. Application of Rheological Techniques to Milk Fat and Milk Fat-Based Dairy Products**

### **22.4.9.1. Milk Fat and Butter**

Rheological studies of milk fat and butter are concerned mainly with objectively measuring spreadability and texture-related properties. Large

deformation tests are particularly useful in this regard, the most common being disc and cone penetration tests and the sectility test (Wright *et al.*, 2001b). In the sectility test, the maximum force required to drive a taut steel wire through the fat or butter sample at constant speed is measured using a UTM-type solids rheometer.

Hardness and apparent yield stress can be calculated from penetration test data, while sectility test data can be converted to a yield stress and a pseudo-Bingham plastic viscosity (Dixon and Williams, 1977).

The AOCS has published a standard method (AOCS Cc 16-60) for constant load cone penetrometry of fats and fat emulsions such as butter (Firestone, 1998).

Large deformation and failure tests can also be carried out in compression, shear and extrusion (through an orifice) using UTM-type instruments, and by means of various imitative techniques (Kawanari *et al.*, 1981; Harnett, 1989; Rohm and Weidinger, 1991; Wright *et al.*, 2001b). Uniaxial compression, which is simple and rapid, is a common test. The resulting force-strain curve can be analyzed to give a value of the ratio of peak force to the work done to peak force, which provides a good way of discriminating between different butter samples. There is some relationship between peak force and sectility force (F. van de Ven, personal communication).

A study (IDF, 1981) in which disc penetrometry, cone penetrometry, extrusion rheometry and sectilometry were compared as ways of measuring butter firmness (an empirical property) concluded that constant speed cone penetrometry was the best method in terms of ease, speed and cost, though reproducibility was not as good as for sectilometry. However, the latest standard for measuring butter firmness, ISO 16305/IDF 187: 2005 (ISO, 2005), is based on sectility measurement. Strangely, the standard specifies a wire cutting speed of 1.0 mm/s, almost 17 times slower than the speed found by Rohm (1992) to be necessary for optimal agreement between sectilometric and sensory evaluations of butter firmness.

The texture and spreadability of butter ultimately depend strongly on its microstructure (Wright *et al.*, 2001b). This structure can be probed using static and dynamic measurements of linear viscoelastic properties in compression and shear using suitable geometries such as cone and plate (shear), parallel disc (shear), parallel disc (compression), and parallel plate (in the form of the parallel rectangular plate viscoelastometer, designed for creep testing in shear) (Harnett, 1989; Rohm, 1993a,b; Narine and Marangoni, 1999; Wright *et al.*, 2001b).

Rohm (1993a) obtained agreement between values of the elongational viscosity of butter measured using creep testing and those obtained using lubricated squeezing flow rheometry, as expected from theory.

References to the literature on the measurement of the textural properties of milk fat fractions, mostly by empirical methods, were tabulated by Kaylegian and Lindsay (1994).

The rheological properties of milk fat and butter, and methods of measuring these properties, were reviewed by Wright *et al.* (2001b).

#### 22.4.9.2. Cheese

Cheeses range from very soft to semi-solid to very hard, and display a very wide spectrum of rheological behavior. Consequently, virtually the whole gamut of rheological test methods already described, and many empirical tests, are applied to cheeses (Gunasekaran and Ak, 2002).

Cheese structure and fracture behavior, and texture-related properties, can be investigated using large deformation and failure testing in compression, shear, tension, torsion or bending. Tension testing is relatively rare because of the difficulty in gripping the sample satisfactorily. Torsion testing is also rare because sample preparation is time-consuming, and the test has a real advantage only for samples that fail at large strains; cheeses suitable for milling into the required capstan shape are relatively brittle, with small failure strains.

Viscometry (using coaxial cylinder, cone and plate and vane rotational viscometers, and controlled stress capillary viscometers) can be used for soft cheeses. Lubricated squeezing flow rheometry is particularly useful for measuring cheese meltability.

Static and dynamic linear viscoelastic measurements are used to gain insights into the relationships between cheese structure and rheological behavior. Non-linear viscoelastic measurements have been used to a relatively small degree to measure the response of cheese to large deformations.

Many empirical, mainly imitative, tests have been designed to measure texture related rheological behavior, fracture properties, melting properties and stretchability.

Compression testing in a UTM-type solids rheometer, especially in the form of the TPA test, is the most common mode of rheologically testing cheeses of which self-supporting cylindrical samples can be prepared; sample preparation is straightforward, the test itself is simple, and valuable information about texture-related attributes is obtained (IDF, 1991; P. Watkinson, personal communication). TPA testing in penetration mode is used for semi-solid cheeses (P. Watkinson, personal communication).

Measurement of the rheological properties of cheese is described in detail by IDF (1991), Gunasekaran and Ak (2002) and O'Callaghan and Guinee (2004).

### 22.4.9.3. Cream

The rheology of cream may be studied using any of the viscometer geometries described above, under controlled shear rate, controlled shear stress or oscillatory shear conditions. Glagovskaia (2000) carried out an extensive study of cream rheology using coaxial cylinder, cone and plate and vane viscometry under controlled stress conditions, dynamic oscillatory testing in shear using the cone and plate geometry, dynamic testing in squeezing flow between parallel discs, and empirical measurements with a Ubbelohde glass capillary viscometer. The dynamic squeezing flow tests were carried out using a CSIRO Micro-Fourier Rheometer. In this instrument, the sample is subjected to controlled small amplitude oscillation, normal to the planes of the discs, with a motion that resembles band-limited random noise. The complete signal spectrum is analyzed simultaneously using Fourier techniques to yield values of the viscoelastic properties complex modulus and complex viscosity (CSIRO, 2005).

### 22.4.9.4. Ice Cream

The rheological properties of ice cream are greatly influenced by its microstructure, which can be studied using dynamic oscillatory testing. Temperature sweep oscillatory testing (sometimes called oscillatory thermo-rheometry) is particularly useful, as the sensory properties of ice cream depend to a large extent on the changes in microstructure and rheological properties that occur during melting (Wildmoser *et al.*, 2004). Ice cream hardness can be determined using penetrometry (Marshall *et al.*, 2003).

The fundamental rheological properties of unfrozen ice cream mix may be studied in the same way as those of cream. Simple highly empirical methods of measuring mix viscosity are commonly used in ice cream manufacture for control purposes (Bhandari, 2001).

### 22.4.9.5. Milk Chocolate

Simple empirical viscometers of the orifice and falling ball types, and the controlled shear rate McMichael coaxial cylinder viscometer, have been used traditionally in the chocolate industry. Sophisticated rheometers are now being used increasingly because the economic pressure to reduce the cocoa butter content of chocolate has generated a need for a greater understanding of chocolate rheology (Minifie, 1999).

## 22.5. Density

The density of fat is most commonly measured at a specified temperature at which the fat is completely molten.

Density *per se* is measured by weighing the sample in a volume-calibrated pycnometer or density bottle using, for example, the IUPAC standard method 2.101 (Paquot and Hautfenne, 1987).

Density is also measured in terms of the specific gravity and the weight of a litre of product in air. The latter is a property of importance in international trade in fats and oils (Firestone, 1998).

In AOCS Official Method Cc 10b-25 (Firestone, 1998) for determining the specific gravity of solid fats at 25°C, the specific gravity of an ethanol-water mixture, in which a small solid sample of the fat neither sinks nor floats, is measured using a Westphal balance (which utilizes the Archimedes buoyancy principle). The specific gravity of the fat sample is equal to this measured value.

The litre weight in air of molten fat is measured using a pycnometer. Standard methods are provided by the AOCS (AOCS Official Method Cc 10c-95, Firestone 1998) and by the British Standards Institution (BS 684-1.1: 2000, BSI, 2000). At temperatures at which the density of water is 1000 kg/m<sup>3</sup> (0–10°C; Cooper and Le Fevre, 1969), the liter weight in air is identical to specific gravity. The liter weight in air becomes increasingly greater than specific gravity as temperature increases above 10°C; the difference between the two is 4.4% at 100°C.

## 22.6. Electromagnetic Properties

### 22.6.1. Refractive Index

The refractive index of an oil or melted fat is defined for practical purposes as the ratio of the speed of light in air to the speed of light in the sample (Rossell, 1986). The difference between these speeds results in light entering the sample from air, or indeed from any medium of different refractive index, being refracted.

The most common instrument for measuring refractive index is the Abbé refractometer, which utilizes the phenomenon of total internal reflection. In this instrument, described in detail by Rheims *et al.* (1997), the sample is sandwiched as a thin layer between an illuminating prism and a measuring prism, both made of glass with a high refractive index (higher than that of the typical sample). The surface of the illuminating prism nearest to the light source is ground, causing diffuse light to strike the interface between that prism and the sample at many angles of incidence. At incident angles lower than  $\arcsin(n/N)$ , where  $n$  is the refractive index of the sample and  $N$  the refractive index of the glass, light incident on the interface between the illuminating prism and the sample is transmitted by the sample, measuring prism and focusing eyepiece. At incident angles higher

than  $\arcsin(n/N)$ , the critical angle, the incident light is totally internally reflected by the illuminating prism. Thus, at the critical angle, the image seen through the measuring prism exhibits a sharp separation between a bright and a dark area. The observation angle is varied (by rotating the prism pair relative to the eyepiece) until the bright–dark boundary is at the point of intersection of a reticle, usually in the form of an X, seen through the eyepiece of the instrument. Sample refractive index is then read from a fixed scale (observable through a second eyepiece) graduated in refractive index (rather than critical angle).

Measurement temperature must be controlled closely, and the oil sample must be optically clear and free of water.

Standard methods for measuring the refractive index of oils and fats are published by IUPAC (standard method 2.102, Paquot & Hautfenne, 1987), the American Oil Chemists' Society (AOCS Official Method Cc 7–25, Firestone, 1998) and the International Standards Organization (International Standard 1739-1975 (E), ISO, 1975). The last specifically applies to the measurement of the refractive index of the fat from butter, and was developed jointly with the International Dairy Federation and the American Oil Chemists' Society.

### 22.6.2. Color

The Lovibond tintometer method described in AOCS Official Method Cc 13e–92 (Firestone, 1998) is the accepted international standard for measuring the color of animal and vegetable fats and oils, and is widely used in most countries other than the United States (O'Brien, 2003). It is based on the British Standard/ISO method BS 684–1.14:1998 ISO 15305:1998 (BSI, 1998a). The method is a subjective one in which the color of light from a standard source as seen through a specified depth of oil is matched with light from the same source transmitted through a selected set of standard colored glass slides, the two light beams being viewed side by side in a telescopic eyepiece (Rossell, 1986; McGinley, 1991). The standard slides are red, yellow, blue and neutral. The slides of any one color are additive: for example, two 5B slides will give the same blue color as one 10B slide. A color match is obtained using red and yellow slides only if possible. Blue slides are used only if necessary. Results are expressed in Lovibond units (e.g., 5R 50Y), and the path length of light in the oil sample must be reported (McGinley, 1991).

The sample must be completely liquid, optically clear, dry and bright. Thus, the color of butter is actually determined on the extracted milk fat (Keen and Udy, 1980). The AOCS method 13e–92 requires that if a sample is not liquid at room temperature, it must be heated to a temperature 10°C above its clear melting point. The operator must not be color-blind.

Lovibond tintometer color can also be measured using objective automated instruments. In one version, the intensities of three light beams (red, yellow and white) transmitted by the oil are measured by photoelectric cells, and the results displayed as red and yellow color readings. The white light beam acts as a reference beam, and allows compensation for variation in the intensity of the light source (Rossell, 1986). AOCS Official Method Cc 13j–97 (Firestone, 1998) specifies how an automated tintometer should be used. However, this standard is valid only for refined oils.

In the United States, the Wesson tintometer method predominates. This subjective method, which is described in AOCS Official Method Cc 13b–45 (Firestone, 1998), is experimentally much the same as the Lovibond method, but uses only red and yellow filters, and specifies the yellow number to be used with any selected red number; the method is thus designed to measure the redness of the sample, and is more limited than the Lovibond method.

Objective spectrophotometric color measurement, as described in AOCS Official Method Cc 13–50 (Firestone, 1998), gives results that are in general highly correlated with Lovibond color, although wide discrepancies occur with some oils (O'Brien, 2003). Method Cc 13–50 is stated to be applicable to cottonseed, soybean and peanut oils, and requires revision to accommodate the use of double beam spectrophotometers. Its applicability to milk fat is not known.

IUPAC also provides a standard spectrophotometric method (standard method 2.103; Paquot and Hautfenne, 1987). The results are reported as transmittance values at specified wavelengths or as a plot of transmittance against wavelength.

The spectrophotometric approach has been used to measure the color of milk fat fractions and butter (Norris et al., 1971; Keen and Udy, 1980; Keen, 1984).

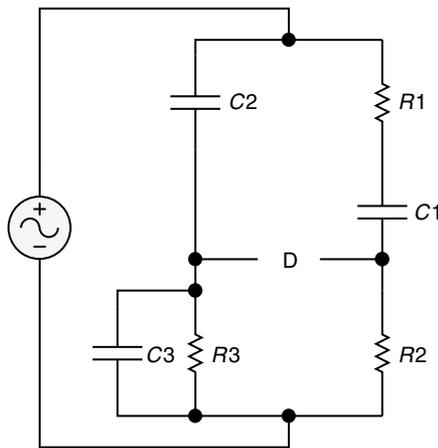
Commercial colorimeters that measure color by analyzing light reflected from the sample surface are readily available (e.g., Konica Minolta, 2005).

### 22.6.3. Dielectric Properties

Measurement of dielectric properties can be used to measure the moisture and/or salt contents of butter and cheese (Parkash and Armstrong, 1969; Parkash, 1970; Bosisio and Huy, 1976; O'Connor and Synnott, 1982; Fagan *et al.*, 2004). Dielectric properties (permittivity,  $\epsilon'$ , loss factor,  $\epsilon''$ , and loss tangent,  $\epsilon''/\epsilon'$ ) can be measured by a variety of techniques that can be grouped into lumped circuit methods, for frequencies lower than  $10^8$  Hz, and distributed circuit methods for higher frequencies (Hass, 1996). The lower frequency range includes mains electricity and industrial radio frequencies, while the higher includes industrial microwave frequencies.

Lumped circuits are typically of the four-arm bridge type, commonly the Schering bridge (Figure 22.10), and are evolutions of the four-arm alternating current bridge counterpart of the well-known direct current Wheatstone bridge (used for measuring an unknown resistance). In the simplest bridge circuit, one arm incorporates the unknown impedance, and the bridge is brought into a null balance (no current flowing) by varying the impedance of one of the other three arms, all of known impedance. For measuring dielectric properties, a test parallel plate capacitor forms part of one arm of the bridge, and its impedance is measured when the sample and then air completely fills the space between the plates. Sample permittivity and loss factor can be calculated from the impedance data obtained (Scaife *et al.*, 1971).

Resonating lumped circuits are alternatives to the use of bridge circuits. These involve the injection (by various means) of a voltage or current into an inductor-tuning-capacitor resonant circuit and the measurement of the resulting voltage across the tuning capacitor at resonance. The impedance of a test capacitor (filled with the sample and then with air), connected across the tuning capacitor of the resonant circuit, is measured by adjusting the latter to restore the resonance that existed in the absence of the test capacitor. An unknown impedance is calculated from the change in the capacitance of the circuit's tuning capacitor and the change in voltage across it (Scaife *et al.*, 1971). O'Connor and Synnott (1982) used a resonance method to measure the seasonal variation in the dielectric properties of butter.



**Figure 22.10.** Schering bridge circuit. The capacitance being measured (the test capacitance) is represented by  $C1$  and  $R1$  in series.  $R3$  is a fixed resistance. Balance is obtained by adjustment of  $C3$  and either  $C2$  or  $R2$ .  $D$  is the detector.

Further lumped circuit options include circuits that allow accurate measurement of the current in, and voltage across, the unknown impedance (thus allowing calculation of that impedance), and the auto balancing bridge (Agilent Technologies, 2003).

Distributed circuit methods use coaxial lines, waveguides and resonant cavities at microwave frequencies. The circuits are designed for measuring an attenuation factor and a phase factor, from which sample dielectric properties can be calculated. The sample may form the dielectric medium between the two conductors of a coaxial line (Scaife *et al.*, 1971), or an open coaxial line is brought into contact with the sample surface (Roussy and Pearce, 1995). Fagan *et al.* (2004) used an open coaxial line method to demonstrate that the moisture and salt contents of processed cheese could be predicted by measuring dielectric properties over a range of frequencies.

In the waveguide method, the sample is contained by the waveguide in the form of a block closely fitting the waveguide's bore.

Resonant cavities are designed to increase the apparent interaction between microwaves and the sample in order to induce measurable attenuation, and are thus particularly useful for measuring the dielectric properties of low loss materials such as fatty foods (Roussy and Pearce, 1995).

Measurements in distributed circuit methods are commonly made with network analyzers (Roussy and Pearce, 1995).

Commercially available impedance measuring instruments are based on circuits described above (Agilent Technologies, 2003).

#### **22.6.4. Electrical Conductivity**

Electrical conductivity is easily determined by measuring the impedance of the sample in the low frequency range (see Section 22.6.3), but at a frequency above the range in which errors arise owing to electrode polarization effects. For example, measurement of the electrical conductivity of cream, which can be carried out by measuring the impedance between a pair of stainless steel or platinum electrodes immersed in the cream sample, should be done at a frequency of  $10^5$  Hz or higher (Lawton and Pethig, 1993).

#### **22.7. Functional Properties**

The general principles of the measurement of rheological properties of milk fat and fat-based products are described in Section 22.4.9. The measurement of other product-specific functional properties, either *ingredient properties* or *end use properties* is described in the following.

### 22.7.1. Milk Fat and Butter

The functional performance of milk fat, milk fat fractions and butter when used as ingredients in complex food products can be evaluated only by means of empirical trials in which products are made and assessed on a laboratory scale using carefully standardized ingredients, equipment, instruments, procedures and reporting of results (O'Brien, 2003). It is commercially important that suppliers of milk fat-based food ingredients be able to demonstrate the efficacy of their products, and be able to supply food manufacturer customers with detailed quantitative advice on how their products can be used to best advantage in customers' products; functional tests provide the means of doing this (O'Brien, 2003).

O'Brien (2003) presents detailed methodologies for assessing fats with respect to their functional properties in cakes, pastry, icing, and flour confectionery and biscuit crèmes.

### 22.7.2. Ice Cream

Air is an important volumetric ingredient of ice cream. The air content is expressed as *overrun*, which is defined as the percentage increase in the volume of the ice cream mix achieved by whipping air into the mix prior to freezing. Ice cream is sold by volume, and its overrun is thus an important property from the regulatory, as well as the product quality, point of view. Overrun is determined by means of appropriate volume measurements (Marshall *et al.*, 2003).

Ice cream *meltdown* at a specified temperature is determined by measuring, as a function of time, the mass dripping from a sample of standard dimensions resting on a screen (Marshall *et al.*, 2003).

### 22.7.3. Chocolate

Particle size is an important property with respect to the sensory quality of chocolate, and in chocolate manufacture. It can be measured using laser light diffraction spectrophotometry (see Section 22.2.11.2), and by a variety of other means such as micrometry, microscopy, wet sieve fractionation, sedimentation and Coulter counting (Minifie, 1999).

### 22.7.4. Whole Milk Powders

The bulk density of powders can be measured very simply by weighing a sample in a volume-graduated container, perhaps after using a standardized tapping procedure to uniformly consolidate the sample.

Powder particle density is measured using an air comparison pycnometer. This comprises two piston-cylinder assemblies of equal size, the pistons of

which are connected to a differential manometer. A known mass of particles is placed in one cylinder. Both pistons are then moved inwards simultaneously (until the second cylinder hits a stop) in such a way that although the pressure in each cylinder increases, the differential pressure remains equal to zero. The particle volume, and hence particle density, is found from the difference in the extents of travel of the pistons, and piston diameter.

Powder flowability is measured empirically as the time for a standardized mass of powder to flow out of a horizontal rotating drum through slits in the drum wall. Drum dimensions and rotational speed are standardized.

Powder particle size distribution is normally measured using laser light diffraction spectrophotometry (see Section 22.2.11.2). The mechanical stability of agglomerated particles can be determined by using this technique to measure the fines created by subjecting the sample to a defined mechanical treatment.

Powder characteristics of a physico-chemical nature, such as *solubility*, *wettability*, *dispersability*, and measures of the “instant” properties of powders (*sludge*, *slowly dispersible particles*, *hot water sediment* and *coffee test*), are determined by a variety of empirical physical tests, some of which incorporate subjective elements. These and the other tests for whole milk powders identified above are described in detail by Westergaard (1994) and Pisecký (1997).

## Acknowledgements

I thank Alan Baldwin, Muffy Colville, Richard Lloyd, Dr Alastair MacGibbon, Frank van de Ven and Dr Philip Watkinson of the Fonterra Co-operative Group Ltd, New Zealand, Ken Mercer of the Institute of Information Sciences and Technology, Massey University, New Zealand and Dr Mike Taylor, Institute of Food, Nutrition and Human Health, Massey University, New Zealand for their help in the preparation of this chapter.

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