RESEARCH

Joseph Dumpler

Heat Stability of Concentrated Milk Systems

Kinetics of the Dissociation and Aggregation in High Heated Concentrated Milk Systems

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Dissertation at TUM School of Life Sciences Weihenstephan of the Technical University of Munich, 2017

ISBN 978-3-658-19695-0 ISBN 978-3-658-19696-7 (eBook)
https://doi.org/10.1007/978-3-658-19696-7

Library of Congress Control Number: 2017954316

Springer Spektrum

Printed on acid-free paper

This Springer Spektrum imprint is published by Springer Nature
The registered company is Springer Fachmedien Wiesbaden GmbH
The registered company address is: Abraham-Lincoln-Str. 46, 65189 Wiesbaden, Germany

Dedicated to my students

Ever tried. Ever failed.
No matter.
Try again. Fail again.
Fail better.
(Samuel Beckett)

Acknowledgements

'Acti iucundi labores' (Cicero), a statement which means 'Completed works are pleasant' when translated to English might be signed by every PhD candidate with regard to his or her finished PhD thesis.

The elaboration and completion of this present PhD thesis was possible due the continuous discussion, untiring advice and supervision of Prof. Kulozik. As the director of the Institute for Food and Bioprocess Engineering of the ZIEL-Institute for Food & Health of the Technical University of Munich, it was him who gave me the opportunity to do research under distinguished conditions, excellent facilities, and all academic freedom possible. He gave me the opportunity to delve into the research field of dairy science and food chemistry. Using this profound background knowledge of dairy chemistry and analysis, it allowed me to unravel a small piece of the unknown. I got a deeper understanding of some correlations and causal relationships and thereby, I could figure out technological approaches to the long standing issue of the heat-induced changes of casein micelles in milk and hand it over to the scientific literature and dairy practice.

Prof. Jörg Hinrichs is thanked for his work on the heat treatment and storage stability of concentrated milk giving direction to my work. In addition, his critical review and examination of this thesis is acknowledged. Prof. Polifke is appreciated for the critical examination of this thesis. Prof. Heiko Briesen is thanked for taking over the Chair of the Examination Committee.

The shaping and implementation of my ideas, suggestions, and plans were enabled by the ongoing support of the members of the institute to the extent presented. Many a time, from the very beginning to my last days at the institute, the team of the institute's own workshop, Christian Ederer, Erich Schneider, and Franz Fraunhofer supported me with their knowledge, expertise, and skills for the construction of parts, assemblies, and installations. The elaboration and continuous improvements of the analytical facilities and methods would not have been possible without the untiring efforts of the staff of the 'HPLC lab' and the 'ELEMENTAR lab' Heidi Wohlschläger, Annette Brümmer-Rolf, Claudia Hengst, Brigitte Härter, and Ilona Hager. The organisational talent and assistance of Mirjana Stulac enabled the large number of pilot plant trials necessary for this work.

Last but not least, my students Eva Gintenreiter, Céline Lang, Sabrina Engelbrecht, Patrick Biebl, Fabian Ständer, Lena Weinzierl, Michaela Neuhauser, Alexander

Hainz, Andrea Machmüller, Laura-Elisa Helgert, Raffael Schuster, Maria Bauer, Barbara Böhmer, Margarita Isolina Valdiviezo Araujo, Rupert Wirnharter, Isabel Muthmann, Immanuele Pinto, Katja Gschaider, Felicitas Peraus, Pascal Birkle, Malou Warncke, and Marie-Therese Dörle are appreciated for their creative and scientific input, assistance, and research work performed at the institute during their seminar, Bachelor's, and Master's theses.
Special and very personal thanks go to the former and current members of the institute who contributed to a pleasant, creative, and relaxed work and leisure time atmosphere, especially my colleagues in the E.25 office over time, Katharina Pruss, Yu Zhuang, Ronald Gebhardt, Iris Schmitz-Schug, Elisabeth Eschlbeck, Sabine Günzkofer and Franziska Kurz.

The research project, from which this work originated, was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support program (Grant number 313.06.01-28-1-74.005-11).

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Abbreviations

Latin symbols

A	Membrane constant	m^2 s kg^{-1}
A	Constant	0.51
AMP	Advanced Maillard products	
BLE	Federal Office for Agriculture and Food (Germany)	
BMEL	Federal Ministry of Food and Agriculture (Germany)	
BSA	Bovine serum albumine	
c	Concentration	mol L^{-1}, M
C5, C6	Molecule with five and six carbon atoms in the backbone, respectively	
C_0	Initial concentration (of protein)	g L^{-1}, %
C_∞	Concentration of protein after infinite heating time	%
CCP	Colloidal calcium phosphate	
CD	Circular dichroism	
CSM	Concentrated skim milk	
C_t	Concentration at time t	g L^{-1}
$C_{T,t}$	Concentration of protein at a discrete temperature and holding time	%
DD	Degree of denaturation	
DSI	Direct steam injection	
D_ϑ	Decimal reduction time at temperature ϑ	s
E_A	Activation energy	kJ mol^{-1}
EDTA	Ethylenediaminetetraacetic acid	
ESL	Extended shelf life	
FAO	Federal Agriculture Organisation	
FMP	Final Maillard product	
f_s	Scaling factor for α_W depending on CSM total solids	-
f_T	Scaling factor for α_W depending on temperature	-
FTIR	Fourier transform infrared spectroscopy	

gal	Galactose	
HCl	Hydrochloric acid	
HCT	Heat coagulation time	s, min
HPLC	High performance liquid chromatography	
I	Ionic strength	mol L^{-1}
J	Flux	m^3 m^2 s
k	Rate constant	s^{-1}
k	Gompertz parameter	$°C^{-1}$
k_0	Apparent rate constant at $\lim_{\frac{1}{T}\to 0} k_T$	s^{-1}
k_{app}	Apparent rate constant	s^{-1}
k_B	Boltzmann constant	$1.3806 \cdot 10^{-23}$ J K^{-1}
KCl	Potassium chloride	
KOH	Potassium hydroxide	
k_{ref}	Rate constant at reference state	s^{-1}
k_T	Rate constant at temperature T	s^{-1}
LOD	Limit of detection	
LOQ	Limit of quantification	
MCC	Micellar casein concentrate	
MPC	Milk protein concentrate	
n	Reaction order	-
N_0	Initial number of microorganisms	-
N_A	Avogadro constant	$6.0221 \cdot 10^{-23}$ mol^{-1}
NaOH	Sodium hydroxide	
NPN	Non-protein nitrogen	
N_{res}	Number of resistant microorganisms	-
N_t	Number of microorganisms at time t	-
P&I	Pipe and instrumentation	
PCS	Photon correlation spectroscopy	
PDB	Protein data base	
PEEK	Polyether ether ketone	
P_i	Inorganic phosphate	
PI_u	Upper limit of the (95%) probability interval	
PPN	Proteose peptone nitrogen	
PS-DVB	Polystyrene divinylbenzene	
Q_{10}	Q_{10}-value	K; °C
R	Universal gas constant	J mol^{-1} K^{-1}
R_1, R_2	Organic residues	
R^2	Coefficient of determination	-
RO	Reverse osmosis	
RP-HPLC	Revesed-phase high performance liquid chromatography	
s	Total solids content of concentrated skim milk	%
S(t)	Survival ratio at time t	-

SMUF	Simulated milk ultrafiltrate	
SMUF-JK	Simulated milk ultrafiltrate according to Jenness and Koops (1962)	
S_P	Sedimentable protein	%
$S_{P,max}$	Maximal amount of sedimentable protein	%
s_{ref}	Reference total solids content of CSM	%
T	Absolute temperature	K
T	Temperature of the Gompertz model	°C
T^*	Reference temperature plus 10 K	K
T_c	Temperature of the inflection point (Gompertz model)	°C
TFA	Trifluoroacetic acid	
thermoph.	thermophilic	
t_R	Reliable lifetime	s
T_{ref}	Temperature at reference state	K
t_{ref}	Reference time	s
TS_0	Initial total solids content	%
$TS_{non\text{-}fat}$	Non-fat total solids content	%
$TS_{non\text{-}fat,\ vc}$	Volume corrected non-fat total solids content	%
TS_T	Total solids content at temperature T	%
UF	Ultrafiltration	
UHT	Ultra-high temperature	
v	Reaction velocity	$s^{-1}\ M^n$
vc	Volume corrected	
VRF	Volume reduction factor	
w/v	Weight per volume	
z	*z*-value	K; °C
z_i	Valency of an ion	-

Greek symbols

$\mu_{\alpha_{w,T}}$	Slope of the regression line of the Arrhenius plot for the characteristic time α_W at temperature T	K
α	activity	$mol\ L^{-1}$
α-la	α-Lactalbumin	
α_W	Weibull parameter	s^{-1}
β-lg	β-Lactoglobulin	
β_W	Weibull parameter	-
γ_i	activity coefficient	-
Δp	Pressure difference	Pa
Δp_{DL}	Presure difference of a deposit layer	Pa

$\Delta\pi$	Osmotic pressure difference	Pa
ϑ	temperature	°C
ϱ_{CSM}	Density of concentrated skim milk	kg m^{-3}
ϱ_{fat}	Density of milk fat globules	kg m^{-3}
Φ_{fat}	Volume fraction of milk fat globules	-

Summary

The necessity to determine the heat stability of concentrated milk originates from the manufacture of evaporated milk and dates back to the late 19th century. It became an issue due to observed particle formation, gelation, and sediment formation in evaporated milk during sterilisation and subsequent storage. Certain batches of evaporated milk, sterilised in cans or glass, and later in HD-PE bottles, showed these instabilities during heat treatment and therefore became defective. Hence, there was an increasing economic interest in the basic understanding and control of the heat stability of the concentrated milk. Since then, dairy science deals with the topic of the heat stability of milk and especially concentrated milk concerning factors affecting the heat stability, methods to predict, and measures to improve it. However, the mechanism of coagulation, factors that cause coagulation and modelling approaches to predict the heat stability of concentrated milk for process optimisation were still lacking.

Individual milieu conditions that affect the heat stability of milk or milk concentrated by evaporation or reverse osmosis have been intensively investigated in the past. These factors comprise the initial pH before heating, the amount of soluble divalent cations, especially soluble calcium, the amount of reactive whey proteins, and the ionic strength in the milk serum. The heat stability of unconcentrated bulk milk at its natural pH is maximal in most cases when dilution is not considered. Attempts to compensate for changes that occur during concentration of milk by removal of water, i.e. changes in pH, ionic strength, soluble calcium, and protein content could not restore the heat stability of unconcentrated milk. Attempts made comprise the addition of bases, citrates, phosphates, and many other permitted and illegal additives for practical applications and research, respectively. Other membrane techniques using porous membranes such as nano-, ultra-, and microfiltration also lead to a reduction in the heat stability of the final concentrate as compared to the unconcentrated milk. However, the overall heat stability of the concentrate increases with increasing compositional similarity of the concentrate to unconcentrated milk. This observation seems plausible. Nevertheless, a prediction of the heat stability of these concentrates in terms of maximum temperature-time combinations for these concentrates under continuous heating conditions without coagulation was lacking.

Technological treatments such as treatments of milk before concentration and heat treatment can affect the heat stability of the final concentrate positively or negatively. Therefore, the combination of processing steps and the choice of processing parame-

ters is decisive to maintain or improve the heat stability of the concentrate. Homogenisation of milk or concentrated milk before heat treatment of the concentrate negatively affects the heat stability. Appropriate preheating of the milk before concentration increases the heat stability of the concentrate. A quantitative estimation of the heat stability expressed as a shift in critical temperature-time combinations had not been performed.

Therefore, the aim of this study was to establish a laboratory test method and a model able to describe the heat stability of concentrated skim milk of various total solids under continuous heating conditions. The limited heat stability of concentrated skim milk of various total solids content should be described quantitatively on lab- and pilot scale. The intention was to be able to design heating processes that maximise microbial inactivation in the concentrates by, at the same time, avoiding heat-induced coagulation of the concentrates. In addition, a description of selected heat-induced changes on casein micelles was also intended. Explanations for the decrease in heat stability by concentration of milk due to changes in milieu conditions should be found and attributed to single components. It was assumed that certain milieu conditions increase the rate of heat-induced changes on casein micelles reducing their colloidal stability and thereby induce coagulation.

The investigation of the heat stability on lab scale was performed using small amounts of concentrated milk filled into small glass tubes tightly screwed and immersed into an oil bath for heating until visual coagulation was observed. Heat coagulation time and temperature were recorded as many samples readily coagulated during heating-up times. The aim of the investigations was to determine the effect of pH, total solids content, preheat treatments, and milk fat on critical temperature-time combinations of the onset of coagulation on lab scale. These critical temperature-time combinations for different total solids content were established by varying the oil bath temperature, i.e. the heating intensity of the samples. The investigation showed that the heat stability of the concentrates progressively decreases with increasing total solids content. A correlation of the heat stability of bulk milk and concentrates produced thereof was possible. The influence of the pH on heat stability decreased with increasing total solids content of the concentrates. The heat stability maximum was at pH 6.7 in all cases. A linear relationship between the logarithm of the coagulation time and the heat coagulation temperature could be established for discrete total solids contents of CSM. This indicated that heat-induced coagulation of concentrated milk follows the principles of formal reaction kinetics. However, an immediate transfer of coagulation temperatures and times to pilot and industrial scale was not possible as the heating profiles of the lab scale system and continuous heating were different.

Direct steam injection was used to perform heat stability testing on pilot scale as it enables isothermal heat treatment of concentrated milk (15-31.5% total solids) due to negligible come-up and cooling times. It was possible to determine the onset of coagulation and the course of coagulation over temperature (117-153 °C) of concentrated skim milk of different total solids content at short holding times (< 13 s). A rapid

increase in the amount of sedimentable heat-induced protein aggregates was observed when certain critical temperature-time combinations were exceeded. These aggregates formed from casein micelles had a size of 3-20 µm, although secondary aggregation to particles of 10-100 µm was pronounced. The size of the aggregates enabled their quantitative separation at 4,000*x*g/10 min in concentrated skim milk. These trials showed a clear correlation between the maximum heating intensity in terms of heating temperature and holding time and the dry matter of the concentrates. A UHT preheat treatment of the milk before concentration resulted in an increase in the heat stability of the concentrate. Higher temperatures or longer holding times became possible without formation of casein micelle aggregates. Heating intensities without coagulation resulted in a remarkable increase in casein micelle size in heated concentrated skim milk. In conjunction with the formation of dissociated casein particles in the range of 20-100 nm, this indicates a marked structural disintegration of casein micelles as a consequence of the heat treatment. This loss of structural integrity of casein micelles could be a preceding reaction to heat-induced coagulation of casein micelles.

Therefore, structural changes of casein micelles and the kinetics of heat-induced aggregation of casein micelles needed to be further addressed. Quantitative ultracentrifugal separation of the different size fractions in direct steam injection heat treated concentrated skim milk was applied. The quantitative separation of the different size fractions was monitored by particle sizing techniques. At the same time, changes in particle size of casein micelles in heated concentrated skim milk of different total solids content were also investigated. The obtained size fractions were analysed for their relative amounts of individual caseins and whey proteins by a refined RP-HPLC method from literature to obtain mechanistic insights into the course and mechanism of heat-induced coagulation of casein micelles. The results showed an accelerated increase of casein micelles with increasing total solids content of the concentrates and increased heating temperature as it was observed for heat-induced coagulation. Coagulated casein was formed by calcium sensitive α_S- and β-caseins. Increasing the heating intensity increased the amount of dissociated casein, mainly κ-casein. Coagulation of casein micelles was observed when 30-35% of κ-casein had dissociated. In addition, the size of casein micelles had doubled at that point. It is assumed that more than one reaction contributes to the destabilisation of casein micelles leading to coagulation.

Furthermore, an extension of the heat treatment trials of concentrated milk and the knowledge obtained concerning the relationship of heating temperature, holding time, and total solids content of the concentrate was intended to derive kinetic parameters (rate constants, activation energies) of heat-induced aggregation of casein micelles. For this purpose, stainless steel tubes were filled with concentrated skim milk (12-33% total solids) and indirectly heated with saturated steam at temperatures from 103 to 131 °C for 0 to 5000 s. After heat treatment, coagulated casein particles were removed by centrifugation. The course of the coagulation process at 27% total solids heated at different temperatures as well as the concentrates of different total

solids content heated at 116 °C could be describe by a Weibullian model. Using this model, critical limits for the heat treatment of concentrated skim milk of different total solids content could be defined. The temperature dependency of the reaction rate could be derived. A basis for the design of non-isothermal heat treatment of concentrates by continuous integration of temperature-time effects was established. The validation of the model was performed by the data obtained from direct steam injection heat treatments at higher temperatures and shorter holding times.

A comparison of the heat stability test results on lab scale with critical temperature-time combinations using direct steam injection heating on pilot scale as well as data obtained by reaction kinetic calculations was performed. This comparison enables to determine the heat stability of different milk concentrates on lab scale and to predict the heat stability of the concentrates under continuous heat treatment.

Further insights into the mechanism of heat-induced coagulation and the relation of the heat-induced dissociation of κ-casein, the increase in casein micelle size, and the heat-induced aggregation of casein micelles in the complex system of concentrated milk appeared to be difficult. Hence, these mechanistic aspects were assessed by using a model system of micellar casein as obtained by diafiltration. This whey protein- and lactose-free casein micelle suspension was manufactured by multiple diafiltration using a simulated milk ultrafiltrate (SMUF). SMUF is a synthetic salt solution that closely resembles the natural milk serum composition. This SMUF solution was developed based on the results of the analysis of ultrafiltration permeate by analytical high-performance ion chromatography which was established for this purpose. In addition, the salt composition and the physical-chemical properties of the SMUF solution depending on temperature and pH were thoroughly characterized. An identical composition and the similarity of the main physico-chemical properties, especially at different working temperatures without crystallisation of calcium phosphate at different working temperatures could be achieved. The dependency of the calcium activity on SMUF composition and pH could be determined by a calcium selective electrode. The region of supersaturation of the SMUF could be predicted by the determination of the pH-dependency of the calcium activity in skim milk.

This diafiltered casein micelle model system using SMUF as a diafiltration medium facilitated a targeted modification of milieu conditions. The interference of the Maillard reaction with analytical determination of caseins by RP-HPLC and the mechanism of heat-induced coagulation was minimised. An increased rate of κ-casein dissociation due to thiol-disulphide exchange reactions with β-lactoglobulin was prevented. The investigations into the effect of pH on the heat-induced coagulation of micellar casein showed that at pH > 6.7 coagulation of casein was very limited, whereas the dissociation of casein, especially κ-casein, from the micelles was very pronounced. With increasing temperature, the amount of dissociated casein increased at the same heating time. Gradual decrease in pH decreased the amount of dissociated casein over holding time at 116 °C. However, there was a significant increase in casein micelle size that could also be observed when soluble calcium was added. The additional increase in ionic strength after calcium addition and a reduc-

tion in pH induced the coagulation of casein micelles to distinct particles. This indicates that in the pH-range of 6.2-7.2, several factors destabilising casein micelles must be present to reduce the colloidal stability of casein micelles and to induce heat-induced coagulation of casein micelles to particles. These factors comprise an increased amount of soluble calcium, increased ionic strength, and a particular heating intensity which are all present in heated concentrated skim milk. Low pH and high soluble calcium content result in the loosening of the internal structure of the casein micelles. The newly-created calcium-sensitive surface of the casein micelles facilitates the aggregation of the casein micelles by exposure of calcium sensitive caseins on the surface of the micelles. The dissociation of κ-casein does not appear to be directly related to heat-induced aggregation, especially at pH < 6.7. However, it should be regarded as a deviation from the original micellar structure of the casein micelles in unheated milk at its natural pH that affects the physical properties of the casein micelles and is likely to foster heat-induced aggregation of casein micelles.

An extension of the term 'heat stability' of the casein micelles is likely to be necessary. A consideration of the dissociation of caseins as well as the loosening of the internal structure of the casein micelles, both related to a loss in native structure is necessary.

The key outcomes of this work can be summarized as follows.

- A kinetic description of heat-induced coagulation of concentrated skim milk on lab- and pilot scale for the determination and calculation of critical temperature-time combinations without coagulation of the concentrates.
- Studies on the dissociation and coagulation of individual caseins in concentrated skim milk heated by direct steam injection.
- The development of a simulated milk ultrafiltrate, e.g. for the purification of casein micelles, to investigate targeted changes in serum composition on heat-induced changes of casein micelles.
- Investigations on heat-induced changes in casein micelle structure and dissociation of caseins in heat treated micellar casein depending on ionic strength, pH and soluble calcium.

Zusammenfassung

Die Notwendigkeit der Bestimmung der Hitzestabilität konzentrierter Milch nahm ihren Anfang mit der industriellen Herstellung von Kondensmilch im späten 19. Jahrhundert. Die Thematik war aufgrund beobachteter Probleme mit der Gerinnung, Sedimentbildung und damit Unbrauchbarkeit einzelner Chargen konzentrierter Milch bei der Sterilisation in Dosen, später in Glas- und HD-PE-Flaschen, von großem wirtschaftlichem Interesse. Seither beschäftigte sich die Milchwissenschaft mit der Thematik der Hitzekoagulation von Milch, insbesondere konzentrierter Milch, deren Einflussfaktoren, Methoden zur Vorhersage und der Verbesserung der Hitzestabilität. Dabei sind trotz intensivster Forschungsarbeit bis heute noch viele Fragen zum Mechanismus, den auslösenden Faktoren der hitzeinduzierten Aggregation der Caseinmicellen und der Modellierung der Reaktion im Sinne der Prozessoptimierung offen.

Die einzelnen Millieufaktoren, die die Hitzestabilität von Milch bzw. mittels Eindampfung oder Umkehrosmose konzentrierter Milch bestimmen, sind sehr intensiv untersucht worden. Zu diesen Faktoren gehören insbesondere der pH-Wert vor der Erhitzung, der Gehalt an löslichen divalenten Kationen im Milchserum, vorwiegend lösliches Calcium, der Gehalt an reaktiven Molkenproteinen und die Ionenstärke im Milchserum. Dabei ist unkonzentrierte Milch mit natürlichen pH-Wert meist am hitzestabilsten, wenn eine Verdünnung nicht in Betracht gezogen wird. Trotz des Versuches, die sich während der Konzentrierung von Milch ändernden Millieufaktoren pH-Wert, Ionenstärke, löslichen Calcium, Proteingehalt teilweise durch Zugabe von Basen, Citrat, Phosphat und vielen weiteren Zusatzstoffen auszugleichen, bleibt die Hitzestabilität konzentrierter Milch im Vergleich zum unkonzentrierten Zustand geringer. Auch andere Membranverfahren der Konzentrierung wie Nano-, Ultra- und Mikrofiltration führen zu einer Verringerung der Hitzestabilität. Dabei wird die Hitzestabilität des Konzentrates umso höher, je weniger dessen Zusammensetzung von der unkonzentrierten Milch abweicht. Dies erscheint plausibel, war jedoch bisher nicht quantitativ in Bezug auf die mögliche Erhitzungsintensität als Funktion der maximalen Temperatur-Zeit-Bedingungen für Konzentrate unter Bedingungen in Durchlauferhitzern ohne Koagulation beschreibbar.

Technologische Einflussfaktoren, d.h. vor allem die Verfahrensschritte vor der Konzentrierung der Milch und Erhitzung des Konzentrates, können sich sowohl negativ als auch positiv auf die Hitzestabilität des Konzentrates auswirken. Die Kom-

bination der einzelnen Verfahrensschritte und die Wahl der Prozessparameter sind daher sehr entscheidend bei der Herstellung haltbarer flüssiger Milchkonzentrate. Die Homogenisierung der Milch oder des Konzentrates vor der Erhitzung des Konzentrates wirkt sich negativ auf die Hitzestabilität aus. Im Falle einer geeigneten Vorerhitzung der Milch vor der Konzentrierung kann die Hitzestabilität gesteigert werden. Eine quantitative Aussage in Form der Verschiebung kritischer Temperatur-Zeit-Bedingungen war bisher nicht möglich. Zudem sind die Prozessparameter zur kontinuierlichen Erhitzung von Milchkonzentraten in Platten- oder Röhrenwärmeübertragern oder mittels direkter Erhitzungsverfahren bisher meist nur empirisch ermittelt und konnten nicht aus der im Labormaßstab ermittelten Hitzekoagulationszeit des Konzentrates ermittelt werden. Eine Berechnung möglicher Temperatur-Zeit-Bedingungen basierend auf reaktionskinetischen Parametern, wie dies für mikrobiologische Inaktivierungs- sowie chemische Effekte möglich ist, war bisher nicht möglich.

Daher war es das Ziel der hier vorliegenden Arbeit, einen Hitzestabilitätstest und eine Berechnungsgrundlage zu schaffen, durch die sich die Hitzestabilität von Milchkonzentraten unter kontinuierlichen Erhitzungsbedingungen, insbesondere von Magermilchkonzentraten, die mittels Umkehrosmose hergestellt wurden, beschreiben lässt. Die begrenzte Hitzestabilität von Magermilchkonzentraten unterschiedlicher Trockenmasse in Bezug auf Erhitzungstemperatur und -zeit sollte quantitativ im Labor- als auch im Technikumsmaßstab beschrieben werden. Dadurch sollte es möglich sein, Erhitzungsprozesse in Bezug auf erwünschte mikrobiologische Inaktivierungseffekte zu optimieren und die Koagulation der Konzentrate während der Erhitzung zu verhindern. Die Beschreibung ausgewählter hitzeinduzierter Veränderungen an den Caseinmicellen war ebenfalls ein Ziel dieser Arbeit. Daraus sollten Erklärungsansätze für die Verringerung der Hitzestabilität von Milch durch die Veränderung verschiedener Millieufaktoren während der Konzentrierung gefunden werden. Es wurde vermutet, dass bestimmte Millieufaktoren zu einer Beschleunigung der hitze-induzierten Verringerung der kolloidalen Stabilität der Caseinmicellen führen.

Die Untersuchungen zur Hitzestabilität im Labormaßstab erfolgte durch die Erhitzung von mit Magermilchkonzentrat befüllten Probengefäßen im Ölbad und der visuellen Bestimmung des Beginns der Koagulation der Konzentrate. Dabei wurde jeweils die Zeit bis zur einsetzenden Koagulation als auch die momentane Temperatur der Konzentrate registriert, da viele Konzentrate bereits während der Aufheizphase koagulierten. Das Ziel der Untersuchung der Hitzestabilität im Labormaßstab war es, den Einfluss von pH-Wert, fettfreier Trockenmasse, Vorerhitzung und Fettgehalt auf die sichtbare Koagulation im Labor-Erhitzungssystem zu bestimmen. Durch die Variation der Ölbadtemperatur sollte der Zusammenhang zwischen der Hitzekoagulationstemperatur und –zeit und diskreter Trockenmassen gefunden werden.

Die Analyse der Hitzestabilität im Labormaßstab ergab zunächst, dass die Hitzestabilität mit ansteigender Trockenmasse der Magermilchkonzentrate kontinuierlich

abnimmt. Eine Korrelation der Hitzestabilität von Sammelmilch und daraus hergestellter Konzentrate war möglich. Der pH-Wert der Konzentrate hatte mit steigender Trockenmasse einen geringer werdenden Einfluss auf die Hitzestabilität, wobei das Stabilitätsmaximum aller Trockenmassen bei einem pH-Wert von 6,7 lag. Zwischen der Koagulationstemperatur und dem Logarithmus der Hitzekoagulationszeit ließ sich für diskrete Trockenmassen ein linearer Zusammenhang darstellen. Dies deutete darauf hin, dass die hitzeinduzierte Koagulation von konzentrierter Milch reaktionskinetisch beschreiben lässt. Eine direkte Übertragbarkeit der kritischen Temperatur-Zeit-Bedingungen vom Labor- in den Pilot- und Industriemaßstab war aufgrund der unterschiedlichen Aufheizprofile des Labor-Erhitzungssystems und einer kontinuierlichen Anlage nicht möglich.

Zur Untersuchung der Hitzestabilität im Pilotmaßstab wurde die Direkterhitzung mittels Dampfinjektion verwendet, die es ermöglicht, bei vernachlässigbaren Aufheiz- und Abkühlzeiten die Konzentrate isotherm zu erhitzen. Damit konnten die Koagulationspunkte sowie der Koagulationsverlauf für unterschiedliche Trockenmassen und kurze Heißhaltezeiten (< 13 s) in Abhängigkeit der Erhitzungstemperatur (117-153 °C) bestimmt werden. Das Überschreiten bestimmter kritischer Temperatur-Zeit-Bedingungen führte zu einem starken Anstieg sedimentierbarer hitzeinduzierter Proteinaggregate. Diese Aggregate aus noch deutlich erkennbaren Caseinmicellen hatten etwa eine Größe von 3-20 µm, wobei die Sekundäraggregation zu Partikeln von 10-100 µm ausgeprägt war. Dadurch waren die hitzeinduzierten Proteinaggregate bereits bei 4000*x*g/10 min vollständig in den erhitzten Konzentraten sedimentierbar. Eine sehr deutliche Korrelation zwischen der maximalen Erhitzungsintensität als Funktion der Temperatur und Zeit und der Trockenmasse der Konzentrate konnte auch im Pilotmaßstab gezeigt werden. Durch eine UHT-Vorerhitzung der Magermilch vor der Konzentrierung konnte die Hitzestabilität der Konzentrate deutlich gesteigert werden, sodass höhere Temperatur-Zeit-Bedingungen zur Erhitzung ohne die Bildung von Proteinaggregaten möglich waren. Erhitzungsintensitäten ohne Koagulation führten zu keiner Sedimentbildung, jedoch bereits zu einer deutlichen Vergrößerung der Caseinmicellen im erhitzten Magermilchkonzentrat. Zusammen mit der Bildung von dissoziierten Caseinpartikeln im Bereich von 20-100 nm deutet dieses auf einen deutlichen Strukturverlust der Caseinmicellen infolge der Erhitzung hin, der die Vorreaktion zur Koagulation der Caseinmicellen darstellen könnte.

Die strukturellen Veränderungen an den Caseinmicellen sowie die Reaktionskinetik der Caseinaggregation sollten daher noch näher untersucht werden. Dazu wurde differentielle Zentrifugation zur quantitativen Trennung der einzelnen Größenfraktionen nach der Erhitzung der Magermilchkonzentrate mittels Direktdampfinjektion verwendet. Die quantitative Trennung der Fraktionen wurde mittels Partikelgrößenanalyse überprüft und die Größenänderung der Caseinmicellen über die Erhitzungsintensität für unterschiedliche Trockenmassen untersucht. Die gewonnen Fraktionen wurden mittels einer weiterentwickelten RP-HPLC Methode auf die relativen Gehalte an einzelnen Caseinen und Molkenproteine analysiert. Daraus sollten Erkenntnis-

se zum Mechanismus der Koagulation und deren Verlauf erhalten werden. Die Untersuchungen zeigten, dass die Vergrößerung des hydrodynamischen Radius der Caseinmicellen ebenso wie die Neigung zur hitzeinduzierten Koagulation mit steigender Trockenmasse der Konzentrate und der Erhöhung der Erhitzungstemperatur zunahm. Koaguliertes Casein bestand vorwiegend aus calciumsensitiven α_S- und β-Casein. Mit steigender Erhitzungsintensität stieg der Anteil an dissoziiertem Casein, wobei vor allem κ-Casein dissoziierte. Ab einem dissoziierten Anteil von 30-35% des κ-Caseins setzte die Koagulation der Caseinmicellen ein. Dabei hatte sich jedoch auch die mittlere Größe der Micellen verdoppelt, sodass mehrere Destabilisierungsmechanismen der Micellen wahrscheinlich sind, die zur Koagulation führen.

Die Erhitzungsversuche von Magermilchkonzentraten und die daraus gewonnenen Erkenntnisse zum Zusammenhang zwischen der Trockenmasse (TM) der Konzentrate und den kritischen Temperatur-Zeit-Bedingungen der Erhitzung sollten noch bis zur Ableitung einer Kinetik der hitze-induzierten Aggregation erweitert werden. Dazu wurden dünnwandige mit Magermilchkonzentrat unterschiedlicher Trockenmasse (12-33% TM) befüllte Edelstahlröhrchen mit Sattdampf auf Temperaturen zwischen 103 und 131 °C zwischen 6 und 5000 s erhitzt und jeweils der koagulierte Anteil an Casein durch Zentrifugation quantitativ abgetrennt. Der Koagulationsverlauf des Konzentrates mit 27% TM, das bei unterschiedlichen Temperaturen erhitzt wurde, sowie der unterschiedlichen Trockenmassen, die bei 116 °C erhitzt wurden, ließ sich zeitabhängig durch ein Weibull-Modell beschreiben. So konnten kritische Grenzen für die Erhitzbarkeit von Magermilchkonzentraten mit unterschiedlichen Trockenmassen, die Temperaturabhängigkeit der Reaktionsgeschwindigkeit abgeleitet werden. Eine Berechnungsgrundlage für nicht-isotherme Erhitzung der Konzentrate durch stetige Integration der Temperatur-Zeit-Effekte wurde erstellt. Die Validierung des Modells erfolgte anhand der Daten zur Hitzeaggregation von Magermilchkonzentraten unter den Bedingungen der Direkterhitzung bei höheren Temperaturen und deutlich kürzeren Heißhaltezeiten.

Ein Vergleich der Ergebnisse der Hitzestabilitätstest im Labormaßstab mit den erhaltenen kritischen Temperatur-Zeit-Kombinationen von Magermilchkonzentraten der Direkterhitzung im Technikumsmaßstab sowie den reaktionskinetischen Berechnungen ermöglicht, die Hitzestabilität von beliebigen Milchkonzentraten im Labormaßstab zu ermitteln und eine Vorhersage bezüglich der Hitzestabilität unter kontinuierlicher Erhitzung zu treffen.

Weitere Erkenntnisse zum Mechanismus der Hitzekoagulation und dem Zusammenhang der hitzeinduzierten Dissoziation von κ-Casein und der Micellvergrößerung mit der Aggregation der Caseinmicellen erschienen in dem komplexen Gemisch Magermilchkonzentrat schwierig. Dies sollte daher mithilfe eines Modellsystems, eines micellaren Caseins, das durch Aufreinigung mittels Diafiltration gewonnen wurde, untersucht werden. Diese molkenprotein- und laktosefreie Caseinmicellsuspension wurde durch mehrfache Diafiltration mit simuliertem Milchultrafiltrat (SMUF), einer synthetischen Salzlösung, die dem natürlichen Milchserum sehr nahe kommt, hergestellt. Das SMUF wurde basierend auf den Ergebnissen der dafür etab-

lierten ionenchromatographischen Trennung der Milchsalze im Milchserum entwickelt. Dieses SMUF wurde bezüglich seiner Salzzusammensetzung und der chemisch-physikalischen Eigenschaften in Abhängigkeit der Temperatur und des pH-Wertes eingehend charakterisiert. Die Zielstellung einer identischen Zusammensetzung und der Gleichheit der wesentlichen chemisch-physikalischen Eigenschaften des SMUF konnte insbesondere für unterschiedliche Arbeitstemperaturen ohne die Kristallisation von Calciumphosphat erreicht werden. Die Calciumaktivität in Abhängigkeit der Zusammensetzung des SMUF und des pH-Wertes ließ sich durch Messungen mit einer calciumselektiven Elektrode nachweisen. Der Bereich der Übersättigung konnte durch den Vergleich mit der pH-abhängigen Calciumaktivität von Magermilch bestimmt werden.

Mithilfe dieses mit SMUF diafiltrierten Casein-Modellsystems war eine gezielte Variation einzelner Millieufaktoren möglich. Die Interferenz der Maillard-Reaktion mit der analytischen Bestimmung der Caseine mittels RP-HPLC und dem Mechanismus der Koagulation wurde minimiert. Die verstärkte Dissoziation von κ-Casein infolge des Thiol-Disulfid-Austausches mit β-Lactoglobulin wurde ebenso unterbunden. Die Untersuchung des Einflusses des pH-Wertes auf die hitzeinduzierte Koagulation von micellarem Casein ergab, dass bei pH > 6,7 die Koagulation sehr stark limitiert war, aber eine Dissoziation eines großen Anteils der Caseine, insbesondere von κ-Casein, beobachtbar war. Mit steigender Temperatur erhöhte sich der Anteil an dissoziiertem Casein bei gleicher Heißhaltezeit. Mit sinkendem pH-Wert ergab sich eine stetige Verringerung des dissoziierten Caseins über die Heißhaltezeit bei 116 °C. Jedoch ergab sich bei niedrigen pH-Werten eine deutliche Vergrößerung der Caseinmicellen, die bei der Zugabe von löslichem Calcium auch beobachtet werden konnte. Die zusätzliche Erhöhung der Ionenstärke nach Calciumzugabe und Verringerung des pH-Wertes induzierte schließlich die Koagulation der Caseinmicellen zu diskreten Partikeln. Dies lässt den Schluss zu, dass im pH-Wert-Bereich von 6,3-7,2 mehrere die Caseinmicelle destabilisierende Faktoren wie ein hoher Gehalt an löslichem Calcium, eine erhöhte Ionenstärke und eine bestimmte Erhitzungsintensität erforderlich sind, um die kolloidale Stabilität der Caseinmicellen so zu schwächen, dass die Aggregation auftritt. Die hitzeinduzierte Aggregation tritt dann ein, wenn all diese Faktoren kombiniert vorliegen, so wie es in Konzentraten aus Mager- und Vollmilch der Fall ist. Niedrige pH-Werte und hohe Calciumgehalte führen dabei insbesondere zu einer Lockerung der inneren Struktur der Caseinmicelle, sodass die neugeschaffene calciumsensitive Oberfläche der Micellen durch die Exposition calciumempfindlicher Caseine die Aggregation begünstigt. Die Dissoziation von κ-Casein erscheint nicht im unmittelbaren Zusammenhang mit der Koagulation, insbesondere bei pH < 6,7, sollte jedoch als eine Abweichung von der originären Micellstruktur der Caseine gesehen werden, die die physikalischen Eigenschaften der Micellen verändert.

Eine Erweiterung des Begriffs der ‚Hitzestabilität' der Caseinmicelle erscheint notwendig, sodass sowohl die Dissoziation von Caseinen als auch die Schwächung

der inneren Struktur, die mit der Koagulation in Zusammenhang steht, berücksichtigt werden.

Die Hauptergebnisse dieser Arbeit lassen sich wie folgt zusammenfassen.

- Eine reaktionskinetische Beschreibung der hitze-induzierten Koagulation von Magermilchkozentraten im Labor- und Technikumsmaßstab zur Bestimmung und Berechnung kritischer Temperatur-Zeit-Bedingungen ohne erkennbare Koagulation der Konzentrate.
- Studien zur Dissoziation und Koagulation einzelner Caseine in konzentrierter Magermilch, die mittels direkter Dampfinjektion erhitzt wurde.
- Die Entwicklung eines simulierten Milchultrafiltrates, z.B. geeignet für die Aufreinigung von Caseinmicellen, um gezielte Veränderungen in der Serumzusammensetzung in Bezug auf hitzeinduzierte Veränderungen an Caseinmicellen zu untersuchen.
- Untersuchungen zu hitze-induzierten Veränderungen der Caseinmicellstruktur und der Dissoziation von Caseinen in erhitztem micellarem Casein in Abhängigkeit der Ionenstärke, pH-Wert und löslichem Calcium.

1 General introduction

The trade of liquid milk and milk powders has increased due to the increasing milk production in dairy farming regions in the world (FAO 2016). Concentration of milk solids by removal of water together with thermal sterilisation of these products offers the opportunity for energy efficient distribution of milk nutrients in liquid form. Thereby, the supply of valuable milk nutrients to climate zones of the world without adequate refrigeration and a climate suitable for dairy farming became possible. This general trend substantiates the need for further development of efficient technologies of milk concentrate thermal processing prior to spray drying or the preservation of concentrated milk solids in a liquid form.

Historically, 'evaporated milk' or 'condensed milk' is one of the oldest, if not the oldest industrially processed dairy product. Evaporated milk, preserved by either in-container autoclave sterilisation (e.g. 115 °C/15 min) or, more recently by continuous ultra-high temperature (UHT; 140 °C/5 s) sterilisation, was one of the first commercially available shelf stable milk products due to its low transportation volume and content of valuable milk nutrients. The process for condensed milk sterilised in tin cans was patented by Gail Borden in the United States and England in the late 19th century (Singh 2004). However, since then, heat and storage stability issues associated with these products have been well known. At the same time, scientific studies on heat stability of milk and concentrated milk emerged in dairy science to solve this problem by a basic understanding of the phenomenon and measures derived from the knowledge gained from the laboratory experiments.

The term 'heat stability' in the context of thermal processing of milk and concentrated milk products for decontamination and preservation refers to the ability of milk to withstand a heat treatment, defined by heating temperature and duration, without coagulation. Coagulation is characterized by the formation of large proteinaceous flocs or dense particles that give rise to a sandy mouthfeel and result in phase separation into a serum phase, protein sediment, and a cream layer in the container during storage if milk fat is present. In these cases, the heat stability of the milk proteins was not sufficient to withstand the heat treatment applied to achieve sufficient microbial inactivation required for preservation. Therefore, we can categorise heat-induced coagulation as an undesired physical destabilisation reaction.

The intensity of the heat treatment applied together with storage conditions determines the shelf life of milk products. The intensity of heat treatment chosen by the

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_1

manufacturer is, on the one hand, guided by consumer expectations concerning shelf life and possible storage conditions such as ambient storage. On the other hand, chemical and physical changes in milk during heat treatment need to be minimised to maintain nutritional value, appearance, taste, and flavour of the milk.

The optimisation of thermal processes is most often performed by the determination of the kinetics of desired and undesired changes in milk within a certain milk system. The kinetics and kinetic parameters of microbial inactivation of microorganisms and bacterial spores in milk and other foods have been properly characterized (van Asselt and Zwietering 2006). The kinetics of heat-induced chemical degradation reactions in milk have also been the subject of numerous studies (Kessler and Horak 1981; Fink and Kessler 1986; Dannenberg and Kessler 1988; Morales et al. 1995; Saint Denis et al. 2001; Claeys et al. 2003). Calculations based on formal reaction kinetics maximising microbial inactivation while, at the same time, maintaining a maximum of nutrients were possible and resulted in the development and implementation of the UHT technology. The heat stability of unconcentrated milk in the range of 15-20 min at 140 °C permits the manufacture of sterilised milk by UHT or in-container sterilisation without noticeable changes in colloidal stability of proteins in milk. The heat stability of concentrated skim milk at 18-20% total solids without further treatment was found to be barely sufficient for sterilisation at 120 °C (Singh 2004). The dependency of the heat stability on the total solids content of the concentrate is yet unknown. Despite of numerous studies in heat stability, the kinetics of heat-induced coagulation of milk proteins in milk and concentrated milk useful for calculations to optimise process conditions remained unknown. Therefore, especially the UHT heat treatment of concentrated milk has been based on empiricism (Muir 1984; Smith and Malmgren 1999).

1.1 Composition of milk and its implication on the heat stability

In addition to heating, milk is most often converted into dairy products by more processing steps. These processes will change its physical state and/or composition in terms of its main components, the major milk proteins, i.e. caseins and whey proteins, lactose, milk fat, milk salts, and water. Therefore, we refer to a 'milk system' containing casein micelles when reporting on milk modified by technological processes. Milk is called 'skim milk' after removal of milk fat by centrifugation. The overall composition of unconcentrated bovine milk is shown in Tab. 1–1.

Concentration of milk by evaporation or reverse osmosis (RO) leads to the increase in concentration of all dry solids of milk by removal of water. Concentration and fractionation of milk by porous membranes like micro- and ultrafiltration in combination with diafiltration leads to the selective enrichment of proteins of milk.

It is therefore useful to provide a brief introduction into the system milk. Otherwise, it would be difficult to fully understand the issues associated with heat stability which was investigated as the key challenge of the processing of concentrated milk systems.

Tab. 1–1: Composition of one kilogram of bovine milk (Töpel 2016).

Component	Amounts	Units	Component	Amounts	Units
Organic constituents			*Organic acids*		
Lipids	30–45	g kg^{-1}	Citric acid	1750	mg kg^{-1}
Proteins	32–36	g kg^{-1}	Lactic acid	34–104	mg kg^{-1}
Caseins	26–30	g kg^{-1}	Ammonia	2-12	mg kg^{-1}
α_{S1}-Casein	10.3–11.9	g kg^{-1}	Urea	100	mg kg^{-1}
α_{S2}-Casein	2.6–3.1	g kg^{-1}			
β-Casein	9.9–11.9	g kg^{-1}	*Inorganic constituents*		
κ-Casein	3.3–3.5	g kg^{-1}	Water	860–880	g kg^{-1}
Whey proteins	6.0–6.2	g kg^{-1}	Cations		
β-Lactoglobulin	3.1–3.5	g kg^{-1}	Calcium	1000–1400	mg kg^{-1}
α-Lactalbumin	1.2–1.3	g kg^{-1}	Magnesium	100–150	mg kg^{-1}
BSA	0.4	g kg^{-1}	Sodium	350–600	mg kg^{-1}
other	1.9–2.3	g kg^{-1}	Potassium	1350–1500	mg kg^{-1}
Carbohydrates	46–48	g kg^{-1}	Anions		
α-Lactose	17.7–18	g kg^{-1}	Chloride	800–1400	mg kg^{-1}
β-Lactose	29–30	g kg^{-1}	Phosphate	2100	mg kg^{-1}
Glucose	50	mg kg^{-1}	Carbonate	200	mg kg^{-1}
Galactose	20	mg kg^{-1}	Sulphate	100	mg kg^{-1}

1.1.1 Caseins

The caseins are the main milk protein fraction comprising approximately 80% of all milk proteins. The caseins are classified as phosphoproteins, which is due to their ester-phosphate groups within the amino acid sequence. In milk, 95% of this protein fraction is organised in micellar form as so-called 'casein micelles'. The term 'casein micelle' originates from one of the first structural models of the quaternary structure of these colloidal particles of caseins in milk. Caseins were assumed to contain hydrophilic and hydrophobic regions, especially κ-casein, and therefore tend towards micelle formation in analogy to an oil droplet stabilised by emulsifiers. Already in the 19th century, casein micelles could be concentrated by a microporous Pasteur-Chamberland-porcelain filter which was an indication for their colloidal nature. About 94% of the casein micelle dry matter is formed by the four main caseins which were denoted as κ-casein, α_{S2}-casein, α_{S1}-casein, and β-casein. The different genetic variants of the caseins differ in the number of ester phosphate groups and some amino acids within their primary sequence (McSweeney and Fox 2013). The quantitative ratio of κ-casein, α_{S2}-casein, α_{S1}-casein, and β-casein in bovine milk is approximately 1.3 : 1 : 4 : 4 (Walstra 1990).

Compared to the calcium-sensitive α_S- and β-caseins, κ-casein is insensitive to the relatively high level of soluble calcium within the serum phase of milk. There is con-

sensus about the location of κ-casein. It is assumed to be mainly attached to the outer surface of the casein micelles as disulphide-linked oligomers ('patches') to form a negatively charged electrostatic and steric barrier towards calcium-induced aggregation as a so-called 'hairy layer' (Rasmussen et al. 1992). The net negatively charged C-termini of glyco- an aglyco-κ-casein reach into the serum phase of milk (Walstra 1990; Holt and Horne 1996). κ-Casein is relatively loosely bound to the micelle surface as it readily dissociates from casein micelles upon heating and an increase in pH (Anema and Klostermeyer 1997; Anema 1998). β-Casein is also assumed to be more loosely attached to the casein micelles by hydrophobic interactions as it dissociates from casein micelles under prolonged cold storage (Creamer et al. 1977; Post et al. 2012). The remaining 6% of the micelle dry matter are composed of inorganic calcium phosphate, calcium citrate, and complexed calcium ions. This colloidal calcium phosphate forms a source for bone mineralisation of the neonate and therefore, the casein micelle can be seen as a carrier structure (Holt et al. 2013). Colloidal calcium phosphate is also an essential structural element of the casein micelles in milk. The high heat stability at the natural pH of milk of about pH 6.6-6.8 and the high pH-sensitivity of casein micelles is often attributed to the linkage between phosphoserine containing caseins formed by calcium bridges and linkages by colloidal calcium phosphate within the casein micelles. The remaining internal stability of the casein complex is given by hydrogen bonds, electrostatic interactions, and hydrophobic bonds. The location of caseins within casein micelles, the formation and nature of electrostatic and hydrophobic bonds, especially the role of micellar complexed calcium and colloidal calcium phosphate within the micelles remain two major uncertainties despite of their known importance for the micellar structure.

Caseins were assumed not to show pronounced secondary and tertiary structural elements like folded α-helices or β-sheet structures and therefore denoted as 'rheomorphic' proteins making them very flexible in terms of conformation. They are assumed not to denature upon extensive heating (Holt and Sawyer 1993). Besides the colloidal nature of casein micelles, this was suggested as a further explanation for the high heat stability of casein micelles compared to globular proteins that readily unfold at temperatures > 70 °C (Walstra et al. 1984). However, Curley et al. (1998) have shown that calcium binding to caseins results in the formation of bonds having a higher bond energy and increase in secondary structural elements such as α-helices and turns within the casein micelles. This was found to be in accordance with the hypothesis on the important role of calcium in terms of micelle swelling and hydration. Farrell Jr. et al. (2001) using Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopies could show that bovine β-caseins undergo several conformational changes depending on temperature and the protein structure to become more flexible with increasing temperature when casein polymers are formed from monomeric β-casein.

Hydrated casein micelles show an average hydrodynamic radius of about 120 nm with a range from 50-500 nm and can bind up to 2.0-2.5 g H_2O g^{-1} protein (Walstra 1990; Fox et al. 2015). Due to this strong hydration, casein micelles make up 12-14%

of the volume of milk whereby the relative mass proportion is about 2.5% (Jeurnink and de Kruif 1993; Dalgleish and Corredig 2012a). This strong hydration and the vast amount of up to 300,000 polypeptide chains per micelle has made it impossible to derive precise information on internal structure of casein micelles in milk by imaging techniques. The estimation of the location and bonding of individual casein molecules is based on energy minimised tertiary structures and assembly studies (Kumosinski and Farrell, JR. 1991; Kumosinski et al. 1991b, 1991a, 1993b, 1993a). These models were derived from properties of the casein micelles depending on changes in the milieu conditions, the response towards technological treatments or imaging techniques. Imaging techniques such as electron micrographs, however, rely on sample preparation that could lead to artefact formation which should be kept in mind. Fig. 1-1 shows electron micrographs of casein micelles prepared with different techniques and taken from various authors.

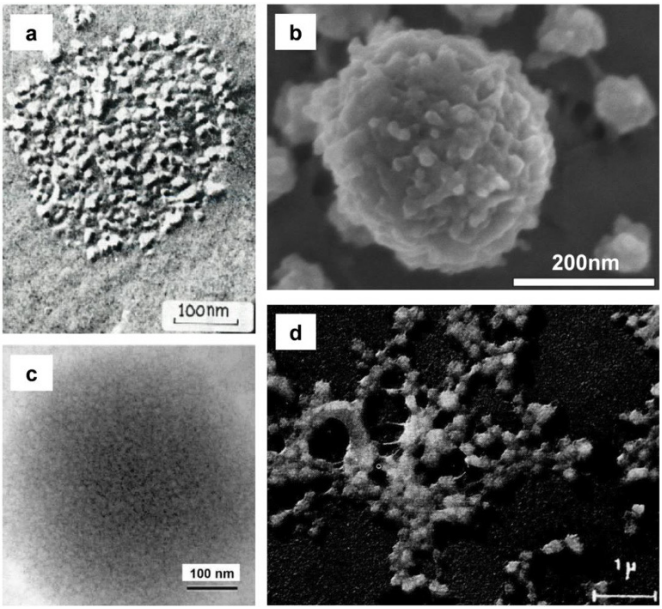

Fig. 1-1: Scanning electron micrograph of a casein micelle in milk after freeze-fractioning (a) from Schmidt (1980). Micrograph of a casein micelle after fixation with glutaraldehyde and osmium tetroxide, dehydration with ethanol, and supercritical drying (b) from Dalgleish et al. (2004). Transmission electron micrograph of a casein micelle stained with uranyl oxalate (c) (McMahon and Oommen 2008). Negative image of a scanning electron micrograph of UHT heat treated condensed milk (d) (Hostettler et al. 1968).[1]

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Fig. 1-1a and b suggest that casein micelles consist of submicellar particles, i.e. protein dense regions within the casein micelle, whereby Fig. 1-1c suggests rather a matrix like structure of the casein micelle, whereby crystalline calcium phosphate is evenly distributed within the casein micelle. Heat treatment of casein micelles in concentrated milk resulted in an increase in casein micelle size and a partial aggregation of casein micelles during UHT heat treatment (Fig. 1-1d). Based on the electron micrographs, Hostettler et al. (1968) stated that the increase in size of casein micelles during heat treatment was proportional to the concentration factor of the concentrated milk. The increase in size was also observed by Carroll et al. (1971). The reason for this increase in size remained uncertain.

Based on experimental results on casein micelle properties, interactions, and imaging techniques, schematic representations of casein micelle internal structures were proposed by several authors and further developed over time. Fig. 1-2 shows the evolution of models proposed over time. The hypothesis of the existence of submicelles is based on experimental evidence. Studies on the self-assembly of caseins in the presence of calcium ions have proven the ability of caseins to form submicelles from monomers depending on environmental conditions (Slattery and Evard 1973). Observations from scanning electron micrographs by Schmidt (1980) (Fig. 1-1a) and the experimental finding that casein micelles dissociate into submicellar particles upon acidification and re-neutralisation or the addition of complex formers have substantiated this hypothesis (Walstra 1990; Fox et al. 2015). Different models based on this hypothesis are shown in Fig. 1-2a–c. The κ-casein-rich submicelles are supposed to be located at the surface of the casein micelles, to limit further growth, and to prohibit spontaneous aggregation of casein micelles in milk. The phosphoserine residues, calcium binding, and colloidal calcium phosphate (CCP) was considered as an elementary building block in all models.

Later models emphasised the role of CCP. The nanocluster model was proposed by Holt et al. (1998) and de Kruif et al. (2012) and the Dual-binding model was proposed by Horne (1998, 2006) who accentuated the role of hydrophobic interactions balanced with electrostatic repulsion as central binding force within the casein micelle. The nanocluster and the Dual-binding model consider the casein micelle as a rather homogenous protein matrix whereby the casein polypeptide chains are oriented towards and anchored in the calcium phosphate clusters (Fig. 1-2d). The presence of κ-casein limits the further growth of casein micelles as it causes chain termination during self-assembly of the casein micelles in the mammary gland.

Models represent a structural simplification of reality and are presented here to give an impression of developments and the current state of knowledge. This was intended to achieve a better understanding of casein micelle properties during processing. It was not intended to argue for one of the models and enter a controversial discussion. It has to be born in mind that any mechanistic explanation on the behaviour of the casein micelles during technological treatments is restricted without the knowledge of the structure of the casein micelles. The capability of these models to

describe technologically relevant properties of the casein micelles is, however, not at all restricted as the models were to a large extent derived from these properties.

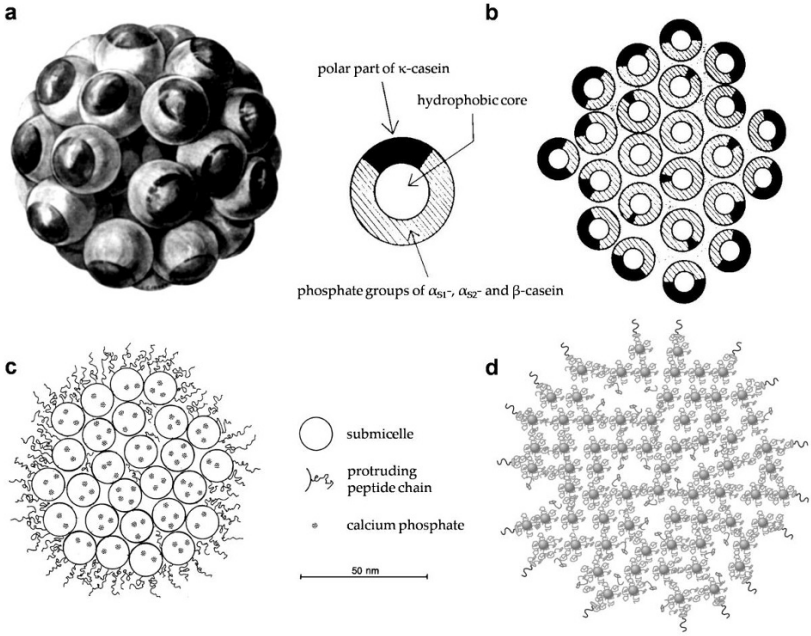

Fig. 1-2: Schematic representation of the casein micelle built up by approximately forty submicelles as depicted by Slattery and Evard (1973) (a). Lighter regions represent hydrophobic regions containing mainly α_{s1}-casein and β-casein. Darker regions represent hydrophilic regions containing κ-casein. A similar model was postulated by Schmidt (1980) whereby colloidal calcium phosphate (CCP) was assumed to fill up the space (speckled areas) between casein micelles (b). In the model of Walstra (1999), CCP was located within the submicelles (c). The model of Dalgleish and Corredig (2012b) is not based on submicelles but rather a matrix structure with CCP nanoclusters surrounded by calcium sensitive caseins that are, in turn, crosslinked by other types of bonds and κ-caseins shielding the micelle surface (d).[2]

The role of κ-casein for the stabilisation of casein micelles against spontaneous or heat-induced aggregation was demonstrated by studies investigating the effect of enzymatic hydrolysis by renneting of milk or the addition of κ-casein to milk on the heat stability at different pH. However, it was observed that excessive pre-renneting was necessary to significantly reduce the heat stability of milk (Fox and Hearn 1978c). No correlation was found between the degree of glycosylation of κ-casein and heat stability (Robitaille and Ayers 1995). Tessier and Rose (1964) found that the ad-

[2] Reproduced with permission from Elsevier Science Ltd, Amsterdam, The Netherlands © 1999, 1980, 1973; Reproduced with permission from Annual Review of Food Science and Technology®, Palo Alto, CA, USA © 2012.

dition of κ-casein resulted in an increase in heat stability over the entire pH-range investigated.

1.1.2 Whey proteins

Compared to casein micelles, whey proteins do not precipitate during acidification to pH 4.6 and they are insensitive to cleavage by chymosin. Therefore, they can be recovered from the remaining serum phase, i.e. the whey, after removal of the caseins during cheese making. Whey proteins account for about 20% of milk protein. The amount of major whey proteins α-lactalbumin and β-lactoglobulin (β-lg) in skim milk is relatively constant. β-Lg accounts for approximately 50% of the whey proteins and 12% of total protein (Fox et al. 2015). Whey proteins are globular proteins that are, compared to casein micelles, far more heat sensitive. The amino acid sequence of these globular proteins determines the formation of extended α-helices in the case of α-lactalbumin (α-la) and β-sheet structures in the case of β-lg. Ribbon type models for the backbone of α-la and β-lg are shown in Fig. 1-3.

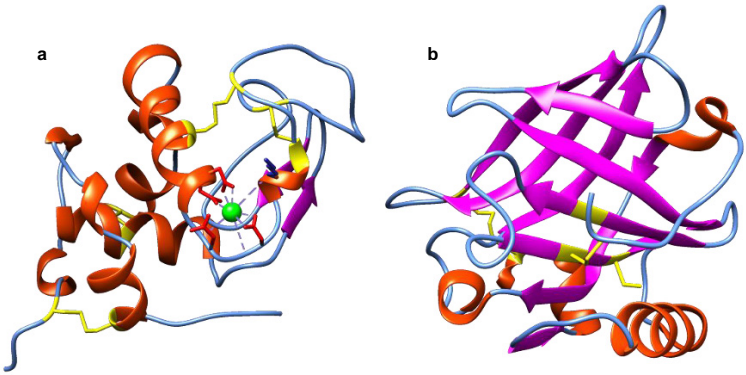

Fig. 1-3: (a) Ribbon type model showing the 3D-structure of α-la using the PDB-code 1HFZ. The green sphere represents a bound calcium ion. Disulphide bonds are marked in yellow. Aspartate residues (red) and the lysine residue (blue) taking part in the calcium complex are indicated. (b) 3D-Structure of β-lg using the PDB-code 5IO5 (Pike et al. 1996). Disulphide bridges and the free thiol group (Cys_{121}) are marked in yellow. Schematic illustrations were produced with the software package UCSF Chimera, candidate version 1.11.2.

The major whey proteins α-la and β-lg differ in their reactivity during heat-induced denaturation. The intramolecular structure of α-lactalbumin contains four covalent bonds formed by disulphide bridges (yellow) and is stabilised by electrostatic interactions of aspartate residues that complex a calcium ion (green). The thermal reactivity of α-la at the natural pH of milk in the absence of β-lg is low. Due to the free thiol group of β-lg that is exposed upon heat-induced unfolding, β-lg is capable to form covalent bonds to other cysteine containing proteins in milk such as α-la and κ-casein

via disulphide exchange reactions at temperatures > 70 °C (Donato and Guyomarc'h 2009). Denatured whey proteins precipitate together with caseins at pH 4.6. The ratio between residual soluble whey proteins after heat treatment as compared to the initial amount present is denoted as 'degree of denaturation' (DD). The interaction of κ-casein with whey proteins when milk or concentrated milk is heat treated affects heat stability as it may foster or prevent casein micelle aggregation depending on milieu conditions, especially the initial pH before heat treatment. Denatured whey protein-κ-casein complexes can form a steric barrier on the surface of the casein micelles against aggregation at pH < 6.7.The pH-range of 6.6-6.8 is the natural pH of unconcentrated milk. In addition, the interaction of κ-casein and whey proteins changes physical parameters such as viscosity, colour, and turbidity as the size of casein micelles and the surface roughness increase due to adsorbed complexes (Anema and Li 2003a). At high pH (pH > 6.7), these complexes will readily dissociate from the micelle surface when milk or concentrated milk is heated resulting in κ-casein-depleted micelles.

The mechanistic aspects of the interaction of whey proteins with κ-casein via thiol-disulphide exchange cannot be observed directly during heat treatment in the turbid milk system, e.g. by laser light scattering. Therefore, structural changes were investigated after heating and cooling of differently heated samples. Fig. 1-4 shows the possible reaction pathways that lead to the formation of whey protein-κ-casein complexes as summarized by Donato and Guyomarc'h (2009).
Denatured whey proteins (mainly β-lg) could at first form primary whey protein complexes, which then associate with whey proteins (A) or form whey protein-κ-casein complexes at the casein micelle surface (B).

The detection of dissociated κ-casein in the milk serum after heat treatment in the ultracentrifugal supernatant of skim milk could then be a result of the dissociation of these whey protein-κ-casein complexes from the casein micelle surface upon prolonged heating (D). However, Anema and Li (2003b) found that the quantities of dissociated κ-casein were found to be larger than the amount of denatured whey proteins after a certain heat intensity in reconstituted skim milk. This suggests the κ-casein dissociation being the preceding reaction before covalent bond formation between κ-casein and β-lg by thiol-disulphide exchange (C). The preferred reaction pathways of association and dissociation might, however, be dependent on processing parameters such as temperature, temperature-time-profiles, and homogenisation as well as milieu conditions like pH, ionic strength, calcium, and lactose. This could explain the differences in the interactions observed between unconcentrated and concentrated milk and the differences in the heat stability pattern of these different milk systems.

In concentrated milk, the natural pH of the concentrates decreases linearly with increasing total solids content (Anema 2009). With decreasing pH, whey proteins were found to increasingly form heat-induced complexes with κ-casein mainly at the surface of casein micelles in unconcentrated milk (Anema 1998). An increasing amount of adsorbed whey proteins increases the heat stability of unconcentrated

milk compared to whey protein free milk or concentrated milk at pH < 6.7. Due to this interaction, whey proteins were shown to influence the pH-sensitivity of heat-induced coagulation of unconcentrated milk, possibly due the formation of a steric barrier of denatured whey proteins towards the aggregation of casein micelles, especially at low pH (Rose 1961a; Tessier and Rose 1964; Fox and Hoynes 1975).

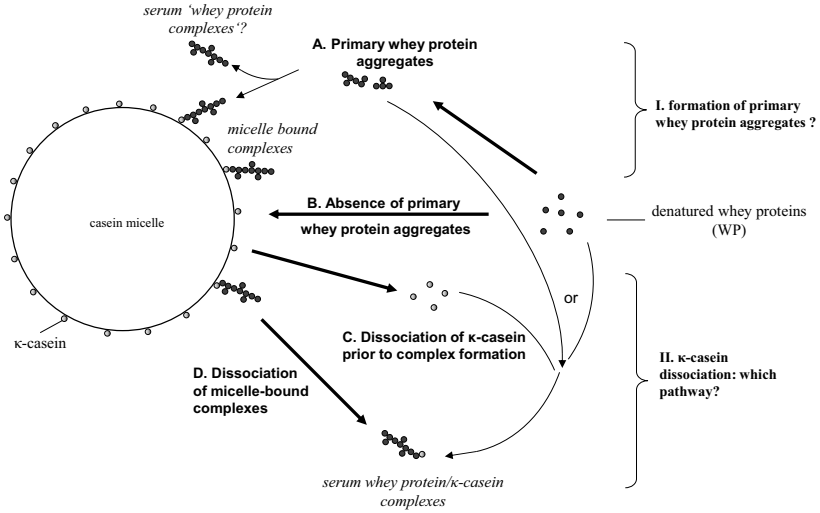

Fig. 1-4: Schematic representation of the proposed pathways of the formation of heat-induced whey protein-κ-casein complexes in heated skim milk. (I) Possible formation of primary complexes of whey proteins in the serum phase of milk. (II) Dissociation of κ-casein for the formation of the serum whey protein/κ-casein complexes (Donato and Guyomarc'h 2009).[3]

In contrast to this, the heat stability of concentrated milk was positively affected when the concentration of whey proteins was reduced and negatively affected over the pH-range of 6.3-7.3 by addition of whey proteins, especially β-lg. This was attributed to the increased rate of κ-casein dissociation upon addition of whey proteins or a crosslinking of casein micelles by reactive whey proteins that cannot be distinguished visually from heat-induced aggregation (Muir and Sweetsur 1978; Newstead et al. 1979).

1.1.3 Lactose

Lactose interacts directly an indirectly with the milk proteins during heat treatment. It comprises about half of the skim milk solids. Lactose is a reducing disaccharide, containing galactose and glucose linked by a β-(1–4) glycosidic bond. Lactose con-

[3] Reproduced with permission from Dairy Science and Technology © 2009, EDP Sciences, France link.springer.com/journal/13594.

tains a hemiacetal structure that is reactive after the opening of the ring structure at the C_4-atom. Prolonged heat treatment of milk at high temperature (> 100 °C for several minutes) results in the formation of early Maillard products. Lactulosyllysine is formed when the aldehyde of lactose reacts with lysine residues within the protein structure. Lactose and available lysine form a Schiff's base that is then further converted to the Amadori product of protein bound lactulosyllysine (van Boekel 1998). This reaction causes a loss in nutritional value of milk products due to the decrease in the amount of the essential amino acid lysine and also results in the modification of charge and glycation (non-enzymatic glycosylation) of milk proteins. The early Maillard product lactulosyllysine is detected either as the artificial amino acid furosine after breakdown of the protein in boiling acid 6-7.8 N HCl or as hydroxymethylfurfural (HMF) after boiling in acetic/oxalic acid overnight (van Boekel 1998). As kinetic studies showed that the chemical reactions can be described by pseudo first order kinetics, i.e. no dependency of the velocity constants on the concentration of reactants, rate constants will not increase by removal of water from skim milk (Fink and Kessler 1986; Morales et al. 1995; Claeys et al. 2003). However, the amount present in the concentrate per volume will increase.

The glycation of caseins might influence the conformation and the colloidal stability of milk proteins (Tan-Kintia and Fox 1999). In addition, the non-enzymatic glycation influences the analytical separation of proteins for quantification by gel electrophoresis and reversed-phase high performance liquid chromatography (RP-HPLC). Pre-peak formation was observed in the chromatograms of UHT milk after prolonged storage (Rauh et al. 2015). It was assumed that a reaction that parallels the Maillard reaction during heat treatment of milk was the most relevant in relation to heat stability of milk. The isomerisation and subsequent thermal degradation of lactose leads to a decrease in pH in milk and concentrated milk due to the formation of formic and lactic acid from lactose. The formation of lactulosyllysine (1) and the formation of HMF and furosine for analytical detection and quantification of the early Maillard product (2) are shown in Fig. 1-5. The breakdown of lactose that leads to the formation of advanced Maillard products and formic acid is shown in Fig. 1-6. This decrease in pH was assumed to negatively affect heat stability due to the reduction of the net negative charge of casein micelles in milk after prolonged heating (Anema and Klostermeyer 1996; Fox et al. 2015). However, a clear correlation between the decrease in pH during heat treatment and the onset and propagation of coagulation could not be established, possibly due to many other heat-induced overlapping reactions taking place during heat treatment.

The amount of lactose will also influence the rate constants of non-enzymatic covalent crosslinking of proteins which results in the reaction products lysinoalanine (from serine and lysine), lanthionine, histidinoalanine and other unidentified reaction products (Zin El-Din and Aoki 1993; Henle et al. 1996; van Boekel 1998). The kinetics of covalent crosslinking of milk proteins are known for whey protein-free micellar casein and the implication of crosslinking on heat stability was discussed (Bulca et al. 2016).

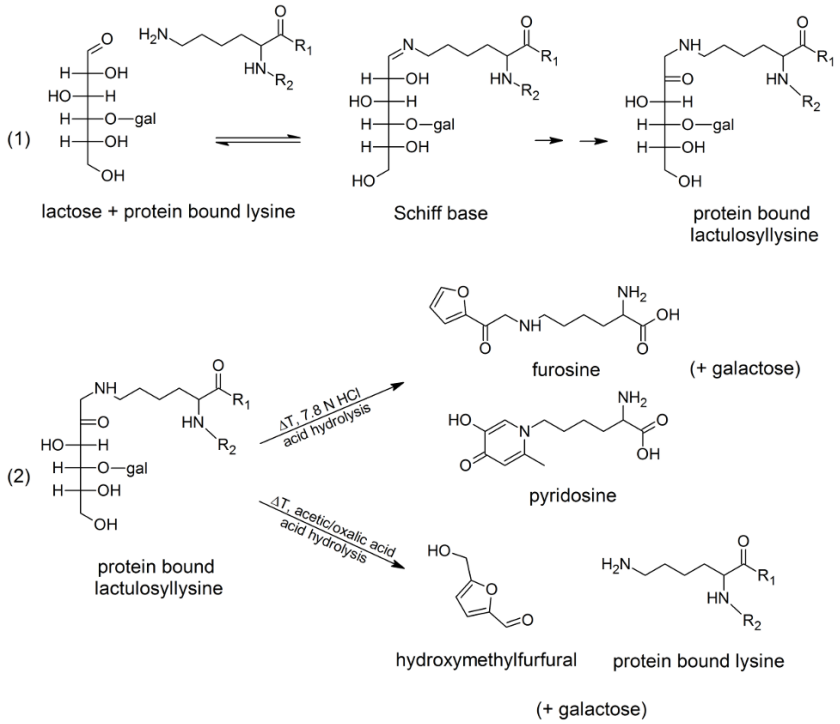

Fig. 1-5: Overview of the early Maillard reaction in milk leading to the Amadori product lactulosyllysine and further breakdown to furosine, pyridosine, and hydroxymethylfurfural by acid hydrolysis for analytical detection (gal = galactose; R_1, R_2 = protein chain) (van Boekel 1998; Fox et al. 2015).

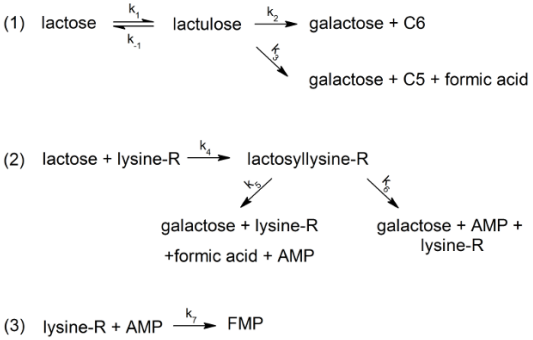

Fig. 1-6: Schematic representation of selected degradation routes of the degradation of lactose in heated milk (AMP = advanced Maillard product; FMP = final Maillard product) (van Boekel 1998).

The kinetics of covalent crosslinking of caseins were approximated by formal reaction kinetics using zero order kinetics up to a polymerisation degree of 50%. A direct relationship between lactose-induced crosslinking of caseins and heat stability was, however, not in the focus of the study and might be hampered due to parallel Maillard reactions taking place when lactose is present. To avoid this, covalent crosslinking of caseins within the casein micelle by chemical or enzymatic crosslinking was performed. This treatment before heating was found to positively affect heat stability of milk and this was ascribed to the reduced rate of κ-casein-dissociation (Singh and Fox 1985b; Huppertz 2014).

1.1.4 Milk salts

Milk salts make up to 7-8 g L^{-1} in skim milk whereby the single constituents can vary considerably, especially in milk from mastitic or late lactation cows. Milk salts are found in milk either soluble in the milk serum or as colloidal complexes associated with caseins within the casein micelle. About 75% of total calcium and 50% of total phosphate is present as colloidal calcium phosphate in milk due to the low solubility of calcium phosphate at the natural pH of milk (pH 6.6-6.8). The equilibrium between the colloidal salts, mainly poorly soluble calcium and phosphate, and the serum phase is called the 'milk salt equilibrium'. Three major principles define the milk salt equilibrium regarding the secretion of milk in the mammary gland.

- Electrical neutrality of the milk salt system.
- An isotonic level of milk salts and lactose in milk as compared to blood.
- The need to form casein micelles from individual caseins.

As a result, there is a positive correlation between the amount of sodium and chloride, a negative correlation between sodium and potassium, and a negative correlation between soluble salts (Na^+, K^+, Cl^-) and lactose present in milk. The amount of total calcium and phosphate in milk is positively correlated as both ions are increasing with increasing casein content in milk due to the increasing amount of CCP. The amount of soluble citrate is positively correlated with calcium as the complex between citrate and calcium is much stronger as compared to phosphate. This explains why the addition of citrate to milk leads to an increase in the amount of soluble casein as the amount of calcium bound to the casein micelle is reduced, either as ion or as CCP stabilising the micellar structure (Gaucheron 2005).

Secretion of milk requires the formation of CCP within the casein micelles to avoid the calcification of the mammary gland. CCP is also considered to stabilise casein micelle quaternary structure and represents a source for bone mineralisation of the neonate. The need to form casein micelles in milk puts constraints on pH and ionic calcium because the self-assembly of caseins to casein micelles will be strongly dependent on pH and ionic calcium (Holt et al. 2013; Fox et al. 2015). Most of the phosphate is present as HPO_4^{2-} and $H_2PO_4^-$ (60:40). Most of the citrate is present as $Citrate^{3-}$ at the natural pH of milk (Fox et al. 2015). The affinity of the phosphate ion to soluble calcium decreases with increasing protonation by a decrease in pH. A decrease in pH

increases the amount of calcium and phosphate present in the milk serum as colloidal calcium phosphate is dissolved when HPO_4^{2-} is protonated to $H_2PO_4^-$ (Frèche and Heughebaert 1989).

The inverse solubility of calcium phosphate in milk with increasing temperature is explained by the increasing affinity of the phosphate ion to calcium with increasing temperature due to a transformation of HPO_4^{2-} to PO_4^{3-} with increasing temperature (Frèche and Heughebaert 1989). Heat treatment of milk results in the reduction of the serum calcium and phosphate level and an increase in colloidal calcium phosphate during heating (Anema 2009). The calcium and phosphate levels in the serum phase are readily restored upon cooling except for heat treatments at > 95 °C for several minutes (van Dijk and Hersevoort 1992). Severe heat treatments like in-container sterilisation (120 °C/20 min) were reported to not only lead to an increase in CCP, but also lead to a structural transformation of colloidal calcium phosphate into another calcium phosphate phase, i.e. another stoichiometric ratio of Ca/P (Holt 1995). The stoichiometric Ca/P_i-ratio of crystalline CCP was assumed to be close to calcium deficient hydroxyapatite or tricalcium phosphate ($Ca/P_i = 1.5$) when the phosphate of phosphoserine residues were excluded from the calculations. The overall ratio of Ca/P including phosphoserines within the casein micelles was found to be close to unity which would suggest CCP to be dicalcium phosphate ($CaHPO_4$). The precipitation of calcium phosphate during heat treatment of milk and especially concentrated milk leads to a decrease in pH as protons are liberated from hydrogen and dihydrogen phosphate to for different forms of crystalline calcium phosphate (Nieuwenhuijse et al. 1988; Gaucheron 2005). In some studies, the precipitation of calcium of calcium phosphate onto casein micelles during heat treatment was assumed to be one of the factors that cause heat-induced aggregation of casein micelles. It was assumed that precipitated positively charged calcium phosphate on the micelle surface reduces the net negative charge of casein micelles and therefore induces aggregation (O'Connell and Fox 2001). Calcium is the limiting factor for heat-induced precipitation of calcium phosphate as the Ca/P_i-ratio in the milk serum is approximately 0.7 independent of milk solids concentration (Anema 2009). Other soluble salts like sodium, potassium, and chloride are sufficiently soluble and are not affected by heat treatments (Fox et al. 2015).

The approximate partition of total calcium in skim milk between casein micelles (colloidal calcium) and the serum phase (soluble calcium) is shown in Fig. 1-7. The colloidal calcium, i.e. calcium bound to casein micelles, can be further divided into CCP and calcium ions complexed by casein micelles via ionic bonds that stabilise casein micelle quaternary structure. Therefore, the caseins within the casein micelle are often referred to as calcium caseinate. The presence of calcium in milk in at least five different forms, mutually in equilibrium, makes it difficult to assess the proportion of a single form of calcium. Changes in pH, temperature, and changes in overall composition will affect multiple forms of calcium at the same time and shift the salt balance to a new equilibrium. Changes in the mineral equilibrium in the milk serum by dialysis or diafiltration against water or resuspension of casein micelles after sed-

imentation by ultracentrifugation in another medium, e.g. distilled water, will lead to a shift in the mineral equilibrium of milk and a redistribution of the different forms of calcium present in the milk serum. Therefore, early studies using these preparative techniques intended to target a single calcium species, i.e. increasing or reducing the amount of CCP in milk serum have to be carefully interpreted in terms of a cause and effect relationship. Excessive removal of CCP will also affect the amount of soluble calcium in the serum. An increase in heat stability observed after removal of CCP might therefore be a result of a reduced amount of serum calcium as well (Fox and Hoynes 1975). The destabilising effect of soluble calcium on the colloidal stability of casein micelles, especially when heated, has long been known. Addition of calcium to milk usually lowers the pH, increases the amount of ionic calcium and CCP, and thereby reduces the amount of soluble phosphate in the milk serum. A few examples of the effect of calcium will be given for illustration. An additional reduction of the pH, in turn, affects the amount of soluble calcium in the milk serum.

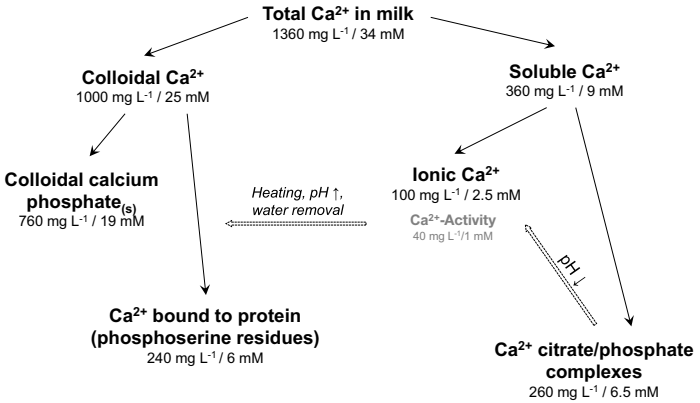

Fig. 1-7: The partition of calcium in skim milk. Approximate amounts for individual species at the natural pH of milk at 20 °C are given; taken from various sources (Walstra et al. 1984; Holt 1995; Vyas and Tong 2004; Gaucheron 2005) and own results. Transitions of individual forms of calcium due to changes in temperature, pH, and water content are shown.

It depends on the aim of the technologist whether casein micelles should be rapidly destabilised to increase the efficiency of processing, protein recovery, and texture or a heat and shelf stable product for many months of storage needs to be obtained. Milk serum composition in terms of pH and calcium will be opposite in these cases. Complete aggregation of caseins (and whey proteins) is intended in the manufacture of so-called co-precipitates of milk proteins from milk, where milk is heat treated at 90 °C for 10-20 min to denature whey proteins and then precipitated at 70-90 °C under addition of 0.03-0.2% calcium chloride (11-72 mM soluble calcium) including slight reduction of the pH if appropriate (Muller 1982). The reason for the precipita-

tion and the mechanisms of destabilisation of casein micelles under these conditions, however, has not much been investigated for these products.

In the case of calcium fortification of fresh milk, there have been numerous studies on the effect of calcium from various sources on the heat stability, i.e. calcium addition as soluble calcium chloride, calcium lactate, and calcium gluconate or as insoluble calcium carbonate, calcium sulphate, and calcium phosphate (Tessier and Rose 1961; Vyas and Tong 2004; Singh et al. 2007; Sievanen et al. 2008; Omoarukhe et al. 2010; On-Nom et al. 2012). The addition of slightly soluble salts had a minor effect on heat and storage stability of milk but insoluble calcium salts had no effect. There is consensus about the destabilising effect of calcium from soluble salts on the colloidal stability of milk. Heat treatment of calcium fortified milk using soluble calcium salts generally led to gel formation or the formation of protein particles that were sedimentable by centrifugation or sedimented during storage of milk. The destabilising effect of calcium on the heat stability of milk was to some extent reduced by the addition of citrates or phosphates to milk before heat treatment due to the complexation of the soluble calcium (Singh et al. 2007; Boumpa et al. 2008; de Kort et al. 2012). Citrates and phosphates are frequently used as stabilisers in the manufacture of evaporated milk. However, the choice of the amount and kind of stabiliser used together with the adjustment of pH by addition of bicarbonates to reduce ionic calcium in concentrated milk is mostly of empirical nature. Stabilisers might negatively affect the shelf life of the concentrate in terms of gelation during storage (Muir 1984). This may be partly due to the complexity of the milk salt equilibrium which makes it difficult to define 'optima' for ratios of individual components and pH. In addition, calcium-induced changes on casein micelles upon heat treatment and their kinetics are not well understood.

Serum calcium in concentrated milk was associated with so-called 'salt-induced coagulation' of casein micelles in concentrated milk and was considered as one of the most important factors reducing the heat stability of concentrated milk compared to unconcentrated milk (Nieuwenhuijse et al. 1991). However, a clear relationship between total serum calcium and overall heat stability of the concentrate has not been established. A clear relationship between the heat stability of unconcentrated milk and concentrates made thereof is also still lacking (Singh 2004).

1.2 Concentration of milk and high heat treatment

Milk is concentrated to increase the nutrient density of milk and to lower the transportation costs. After concentration, the concentrate can be heat treated and/or dried for preservation. Concentrated milk products in the range of 18-20% non-fat total solids are mostly (UHT-)sterilized to prevent microbial spoilage, a product known as condensed or 'evaporated milk'. Higher concentrated milk is mostly spray dried. This is done after concentration of milk by only evaporation or RO in combination with evaporation. The efficiency of the concentration process is increased by using

RO or a combination of RO and evaporation due to the lower energy demand of RO per kilogram of water removed from skim milk.

The heat stability of highly concentrated milk is low and does not allow for sterilisation heat treatments due to severe structural changes like gel or particle formation during heat treatment. However, quantitative limits for the heat treatment of concentrates > 20% non-fat milk solids have not been established. This appears to be relevant when we consider that partial inactivation of microorganisms in dairy products has recently become a much more frequently used and successful technique for preservation. Partial inactivation is used to prolong the shelf life or ensure the microbial safety in the context of extended shelf life (ESL) milk (Mayr et al. 2004; Schwermann and Schwenzow 2008a, 2008b) and to inactivate pathogenic spore formers in liquid milk concentrates, such as *Bacillus cereus* (Stoeckel et al. 2014).

1.2.1 Principles of evaporation and reverse osmosis

During concentration of milk by evaporation or pressure driven RO to remove water from milk, i.e. as vapour or permeate, all milk solids are concentrated. Evaporation is an energy intensive thermal process whereby water is removed under vacuum at elevated temperature in the range of 45-75 °C using multi-stage falling film evaporators (Kessler 2002). Some volatile components like CO_2, ammonia, acetic, and lactic acid is also be partially removed from milk during concentration resulting in slight changes in composition and pH in the final concentrate. The implication of a heat treatment of milk or concentrated milk before the final heat treatment of the concentrate for preservation will be discussed in section 1.4.2. The evaporation process of skim milk is limited by the viscosity of the concentrate and fouling on heat transfer surfaces. A total solids content of skim milk concentrate of 50-65% can be achieved by evaporation (Carić et al. 2009).

RO as applied in dairy industry uses hydrostatic pressure in the range of 2-4 MPa to overcome the osmotic pressure in the concentrate, i.e. the retentate, due to soluble sugars and salts. Pressure is used to reverse the phenomenon of osmosis by hydrostatic pressure. Osmosis describes the physical phenomenon of a pure solvent to dilute solvents containing solutes like sugars or salts when these two solutions are separated by a membrane that allows for the diffusion of the solvent but not the solutes. RO removes water from milk by diffusion through a semipermeable membrane without a phase transition from water to vapour. The water is adsorbed at the membranes surface, diffuses through the membrane, and is desorbed at the other side of the membrane. At the membrane surface, the concentration of the solutes, and therefore the osmotic pressure is further increased by the permeation of water through the membrane and the rejection of solutes. This is denoted as ‘concentration polarisation’. The driving force to overcome the diffusion resistance of the hydrophilic polyamide composite membrane is the net pressure difference Δp. This net positive pressure difference is calculated from the hydrostatic pressure and the osmotic pressure

$\Delta\pi$ of the retentate. The membrane constant A is membrane specific and describes the correlation between the area specific amount of filtrate and the net positive pressure difference as shown in eq. 1.1. Due to membrane fouling and scaling of salts over time depending on the composition of the retentate, the resistance of the deposit layer Δp_{DL} needs to be added to the membrane resistance (Kulozik 1986). The inverse of the membranes constant A can be denoted as a membrane specific 'diffusion resistance' of the membrane.

$$J = A \cdot [\Delta p - (\Delta\pi + \Delta p_{DL})] \quad (1.1)$$

This equation applies when the concentration of solutes in the permeate is negligible as it is the case for RO membranes with a rejection of NaCl > 99%. The net positive pressure difference is reduced with increasing total solids content of the milk concentrate due to an increase in osmotic pressure by salts and lactose. The increasing fouling resistance with increasing total solids content and additional build-up over time further limits the maximum concentration volume reduction factor (VRF). The VRF is calculated by the quotient of the total solids content of the concentrate in relation to the total solids content of the skim milk. A VRF of 2-3.1, i.e. 18-28%, can be achieved by industrial RO considering reasonable runtimes without excessive fouling of membranes (Depping et al. 2017). Industrial membranes are usually run in 'cross-flow mode'. Concentration polarisation of solutes and deposit layer formation on the membrane surface is controlled by tangential flow of the retentate over the membrane surface to increase the convective transport away from the membrane surface (Melin and Rautenbach 2004).

Low molecular weight nitrogen as substances like urea and ammonia can pass RO membranes. A linkage between urea in milk and heat stability has often been discussed (Muir et al. 1979; Pearce 1979; Dalgleish et al. 1987, 1987; Metwalli and van Boekel 1996; Metwalli et al. 1996). Concentrated milk prepared by evaporation as compared to RO has a higher heat load due to the concentration at higher temperatures during evaporation. This might result in partial denaturation of whey proteins and the cross-linkage of especially β-lactoglobulin with κ-casein via disulphide interchange reactions which can affect heat stability (Oldfield et al. 2000; Lowe et al. 2004).

1.2.2 Design of high heat treatments for dairy products

Milk is easily spoilt and contaminated by growth and metabolism of microorganisms or food pathogens as milk is an ideal substrate for microbial growth. Therefore, heat treatment is on the one hand applied to minimize the risk of food-borne diseases from milk consumption and on the other hand applied for elongation of the shelf life of dairy products by a reduction of the initial number of microorganisms. Due to this, heat treatment of milk is the most frequently applied processing step in dairy technology. A high heat treatment can be defined by a heating temperature exceeding 85 °C. The intensity of the heat treatment applied to preserve liquid milk products

can vary widely depending on microbial safety and shelf life requirements, the inactivation kinetics of the target organisms, and other milieu conditions that might inhibit microbial growth such as refrigeration. Pasteurisation (72-75 °C/15-7s) is used for sufficient inactivation of food pathogens and the elongation of the shelf life of milk up to 4-7 days. ESL milk is produced by high heat treatment in the range of 125-127 °C/2-4s for sufficient inactivation of mesophilic sporeformers to a achieve a shelf life of 21-35 days under refrigeration < 8 °C. UHT milk products are sterilised in the range of 135-150 °C/1-10 s to achieve commercial sterility and a shelf life of up to 6 months at ambient conditions.

Kinetic modelling of thermal processing

During thermal processing, milieu conditions, temperature and pressure will decide about the thermodynamics, i.e. the direction a reaction proceeds and the potential between the initial state and the final equilibrium. Kinetics provides additional information about the dependency of rate of a reaction leading to another state depending on the concentration of reactants, temperature/pressure, possibly catalysts, and time.

Depending on the characteristics of a reaction, one can refer to thermodynamically or kinetically controlled reactions. A thermodynamically controlled reaction follows rather fast kinetics towards a new equilibrium state when no barrier is present. A kinetically controlled reaction might proceed at a rather slow rate. The equilibrium is not attained within the time considered or relevant. The composition of the reaction mixture is then determined by the kinetic parameters and the equilibrium is mostly not reached. The nature of the reaction, i.e. the reactants involved and the resulting products, is described by the mechanism of the reaction (van Boekel and Walstra 1995). In complex food systems containing many reactants, the reactions taking place are usually coupled with interfering side reactions and therefore too complex to determine all intermediate products, reactions involved, and a detailed mechanistic description.

In the simplest case, the decrease in concentration of an initially present component A or a product B, e.g. a nutrient and a degradation product, can be determined analytically over time. The conversion rates can be determined when we interpret the resulting rate constants as 'apparent' rate constants to describe the overall course of the reaction.

$$A \underset{-k_A}{\overset{k_A}{\rightleftharpoons}} B \tag{1.2}$$

Using a formal reaction kinetics approach, changes in concentration over time, i.e. reactions of the destruction of a component A or formation of component B, can be described whereby $k_A \gg -k_A$. Eq. 1.3 describes the relation as a differential equation in the case of a destruction reaction of component A in the form

$$v_A = -\frac{dc_A}{dt} = k_A \cdot c_A^n \tag{1.3}$$

and the formation reaction of component B as eq. 1.4

$$v_B = \frac{dc_B}{dt} = k_B \cdot c_B^n = - k_A \cdot c_A^n \qquad (1.4)$$

where v is the velocity of the reaction, c is the concentration of the components, k is the rate constant, and n is the order of the reaction with respect to c. The reaction order n indicates the empirical dependence of the reaction on concentration. The general integration of the differential eq. 1.3 over time *t* for decomposition of a component results in eq. 1.5 for the reaction order being different from unity.

$$\frac{C_t}{C_0} = \left[1 + (n - 1) \cdot k_{app} t\right]^{\frac{1}{1-n}} \qquad n \neq 1 \qquad (1.5)$$

where C_t is the concentration of the component at time t, C_0 is the initial concentration before heating when a thermal reaction is considered, and k_{app} is the apparent rate constant. In the case of a first order reaction (n = 1), eq. 1.3 is integrated to

$$\frac{C_t}{C_0} = \exp(-k_{app} t) \qquad n = 1 \qquad (1.6)$$

The linearization of eq. 1.5 and eq. 1.6 by rearrangement was used to determine k_{app}as the slope of the graphs when the reaction order is iteratively estimated until a straight line is obtained (Kessler 2002).

The inactivation of microorganisms was mostly described by first order kinetics as it was assumed that the inactivation of microorganisms follows the principle of decay whereby the rate constant is independent of the number of microorganisms present at any time *t*. Heat-induced chemical reactions in milk described in literature were described as zero (formation of HMF) (Fink and Kessler 1986), first (formation of lactulose) (Nangpal and Reuter 1990) or second order reaction (destruction of thiamine) (Horak 1980). However, reaction orders were not always consistently described for the formation of a certain component in literature. In some cases reaction orders determined were denoted as 'pseudo-orders' as the determination of the 'true' order of a reaction was impossible due to interfering reactions in complex food systems, especially noticeable after prolonged heating times (Claeys et al. 2003). The denaturation of proteins was found to be either a first order reaction for pure α-la or milk plasmin (Saint Denis et al. 2001) or non-integer (n = 1.5) for the denaturation of whey proteins in milk (Dannenberg and Kessler 1988).

The dependency of the rate constants on temperature is usually approximated by the empirical Arrhenius equation (eq. 1.7).

$$k_T = k_o \cdot \exp(-\frac{E_A}{R \cdot T}) = k_o \cdot \exp(-\frac{E_A}{N_A \cdot k_B \cdot T}) \qquad (1.7)$$

where k_T is the (apparent) rate constant at a certain temperature, k_o is the rate constant at $\lim_{\frac{1}{T} \to 0} k_T = \infty$, R is the universal gas constant, and T is the absolute temperature. Instead of R, the product of the Boltzmann constant k_B and the Avogadro con-

stant N_A could also be used which might be more plausible in the context of liquid foods as $k_B \cdot T$ describes the specific thermal energy of a system.

In order to increase the precision of predictions and to reduce the dependency of E_A on k_o, a reference temperature T_{ref} in the center of the range investigated is introduced. This is due to the fact that the temperature range relevant in thermal processing (and storage) of food is small compared to the absolute temperature range.

$$\frac{k_T}{k_{ref}} = \exp\left[-\frac{E_A}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right] \tag{1.8}$$

where k_{ref} is the (apparent) rate constant at the reference temperature T_{ref}. The rate constants are inversely proportional to times required to achieve a certain effect according to eq. 1.9

$$\frac{k_{ref}}{k} = \frac{t}{t_{ref}} \tag{1.9}$$

where t_{ref} is the time at the reference temperature. The corresponding time until a certain effect is achieved can then be defined as

$$\frac{t}{t_{ref}} = \exp\left[-\frac{E_A}{R} \cdot \left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \tag{1.10}$$

Eq. 1.10 is more commonly written in a logarithmic form as

$$\lg \frac{t}{t_{ref}} = -\frac{E_A}{2.303 \cdot R} \cdot \left(\frac{1}{T_{ref}} - \frac{1}{T}\right) \tag{1.11}$$

Rearrangement of eq. 1.11 yields

$$\lg \frac{t}{t_{ref}} = -\frac{E_A}{2.303 \cdot R \cdot T \cdot T_{ref}} \cdot (T - T_{ref}) = -\frac{1}{z} \cdot (T - T_{ref}) \tag{1.12}$$

with

$$z = \frac{2.303 \cdot R \cdot T \cdot T_{ref}}{E_A} = \frac{10}{\lg Q_{10}} \tag{1.13}$$

where z represents the z-value. The z-value is the increase in temperature required to reduce the time to achieve a certain effect by 90%. The Q_{10}-value is the increase in the rate constants when the heating temperature is increased by 10 K (van Boekel and Walstra 1995). E_A, z-value, and Q_{10} are therefore related numbers that describe the temperature sensitivity of a reaction.

When E_A was determined from experimental results using eq. 1.8, a certain effect in terms of microbial or enzyme inactivation can be defined ($\frac{C_t}{C_0}$ = const.) and so-called 'lines of equal effect' or iso-effect lines can be derived by using eq. 1.12. Examples for these iso-effect lines calculated from data published in literature for unconcentrated milk are shown in Fig. 1-8.

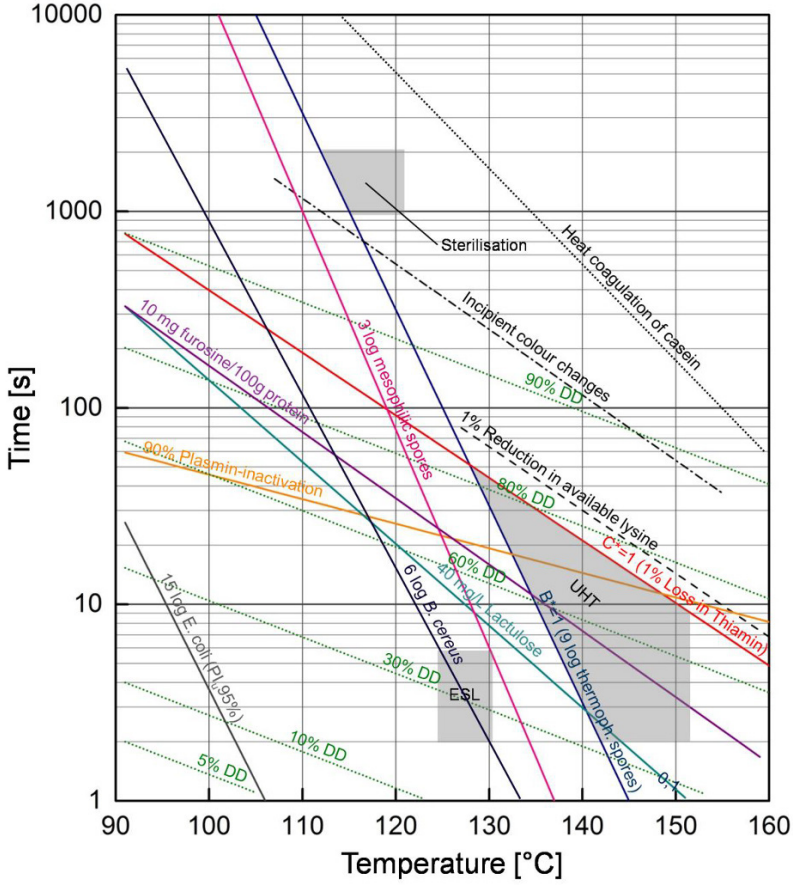

Fig. 1-8: Iso-effect lines for selected microbial inactivation levels of mesophilic spores, thermophilic spores, *B. cereus* (mean D-value), and *E. coli* (95% upper probability interval), denaturation of major whey proteins (DD = degree of denaturation), inactivation of milk plasmin by 90%, destruction of nutrients (lysine, thiamine), formation of heat indicators (lactulose, furosine/lactulosyllysine), changes in colour, and heat coagulation of casein in milk (Kessler 1975; Kessler and Horak 1981; Walstra et al. 1984; Fink and Kessler 1986; Dannenberg and Kessler 1988; Saint Denis et al. 2001; Claeys et al. 2003; van Asselt and Zwietering 2006).

Using these lines, a quantitative definition of working rages to achieve a certain microbial effect and, at the same time, minimal chemical damage on valuable milk nutrients is possible. In order to minimise chemical changes, working ranges were defined at high temperature and short times in the case of ESL and UHT milks. Microbial inactivation is sufficiently achieved whereby chemical changes are reduced to a minimum due to the lower temperature sensitivity of chemical reactions. In practice,

the formation of furosine, lactulosyllysine, and the denaturation of whey proteins can be taken as 'temperature-time integrators' to be able to analytically determine the heat load of a milk sample for process optimisation, legislative purposes, and threshold definition for consumer protection (Morales et al. 2000; Elliott et al. 2005; Mayer et al. 2010; Mayer 2011; Boitz and Mayer 2015). These iso-effect lines also show that the coagulation of caseins in unconcentrated milk is not an issue when milk is sterilised either by in-container sterilisation or UHT heat treatment. However, iso-effect lines for the coagulation of concentrated milk have not been described in literature.

Recent developments in kinetic modelling

In the recent years, formal reaction kinetic approaches were criticized for simplifications made and a higher precision of prediction was desired. Formal reaction kinetic approaches were found to be limited in precision to describe microbial inactivation at low heating temperatures, i.e. slow inactivation rates (Albert and Mafart 2005; Corradini et al. 2006). In addition, the two-step approach of iterative guessing of the reaction order and subsequent determination of the rate constants was found to be outdated in times of high-performance computers. Non-linear regression and the numerical iterative estimation of multiple parameters represent no longer an issue nowadays (Loveday 2016). It was even suggested to waive any relation of parameters and their estimation with mechanistic insights in kinetic modelling of reactions in foods and to use purely (non-linear) empirical models resulting in the best fit to data. These models were shown to be able to predict kinetic data and rate constants (Peleg et al. 2004) or the activation energy (Barsa et al. 2012) similar to mechanistic models due to their mathematical similarity.

However, Loveday (2016) showed that the reaction order and rate constants determined by previous studies using the two-step linear regression could be mostly confirmed in terms of the denaturation kinetics of β-lg by revisiting published data using 2- and 3-parameter non-linear regression. However, some additional precision of non-linear regression was claimed and in addition to parameter estimates, statistical measures of precision were given to describe the goodness of fit of the model.

Mathematical empirical models other than a formal reaction kinetic approach for microbial inactivation were proposed in literature. Albert and Mafart (2005) found that the heat-inactivation of microorganisms could be better described by a modified Weibullian model than by first order kinetics. Microbial 'inactivation curves' most frequently show slow initial inactivation and an increased inactivation rate over time which is attributed to continuous thermal damage on bacterial cell components. Tailing of the curve can also be present, i.e. a highly resistant subpopulation or viable microorganisms or spores that are not inactivated even after prolonged heating. These phenomena can be modelled by using an empirical Weibullian model (eq. 1.14) to describe microbial inactivation.

$$S(t) = \frac{N_t}{N_0} = \exp\left[\left(\frac{t}{\alpha_w}\right)^{\beta_w}\right] \tag{1.14}$$

where S(t) is the survival rate calculated from the amount of remaining microorganisms N_t as compared to the initial number N_0. The Weibull parameter α_w can be interpreted as a characteristic time. The Weibull parameter β_w describes the curvature of the graph, either downward ($\beta_w < 1$) or upward concavity $\beta_w > 1$) (van Boekel 2002). For the case of $\beta = 1$, the Weibullian model is equivalent to formal first order kinetics. The Weibull parameter α_w is then equivalent to the D_ϑ-value in formal first order kinetics as described above. The D_ϑ-value represents the timespan required at a fixed temperature to reduce the population of microorganisms by 90%.

Eq. 1.14 is mostly used in its logarithmic form for the description of microbial inactivation

$$\lg S(t) = \lg \frac{N_t}{N_0} = \frac{1}{2.303} \cdot \left(\frac{t}{\alpha_w}\right)^{\beta_w} \tag{1.15}$$

and can be modified by the introduction of a new parameter, a lower asymptote that allows the model to include the resistant population N_{res} of microorganisms.

$$\lg S(t) = \lg \frac{N_t - N_{res}}{N_0 - N_{res}} = \frac{1}{2.303} \cdot \left(\frac{t}{\alpha_w}\right)^{\beta_w} \tag{1.16}$$

Nevertheless, the introduction of more and more parameters into a model to increase the goodness of fit results in a conflict with the statistical significance of these parameters. Therefore, statistics is required to check if the variation of the dataset obtained by experiments is sufficiently small to justify the number of parameters of the preferred model. In other words, a high variation on the dataset obtained by a poor experimental and/or analytical set-up necessarily results in a very simple model to describe the kinetics of the reaction. In the worst case, not even a linear model can show a significant increase or decrease over time. The decision for as little parameters as necessary is called 'Ockham's razor' (van Boekel 2009). In the case of fast kinetic inactivation of microbial spores at high temperature, the approximation of inactivation data by linear regression becomes increasingly precise compared to the experimental error associated with microbiological methods, i.e. for very low values of α_w, the Weibullian model can be approximated by a straight line. The remaining population of heat resistant spores, accounting for a pronounced tailing of the inactivation data even at high temperatures, is responsible for spoilage of a very small proportion of UHT milk packages. In practice, this phenomenon is referred to as 'commercial sterility'.

Industrial design of high heating processes

High heating processes in dairy industry are designed to kill all viable microorganisms and a sufficient inactivation of bacterial spores to obtain ESL products or 'commercial sterility'. ESL products need to be stored under refrigerated conditions due to the incomplete inactivation of bacterial spores that would germinate and spoil the product when stored at room temperature within a few days. UHT processes were designed to sufficiently inactivate bacterial spores that are able to germinate under ambient storage conditions of the final dairy product.

These continuous heating processes can be divided into categories by the mode of heat transfer to the product and further by the construction of the equipment as shown in Fig. 1-9.

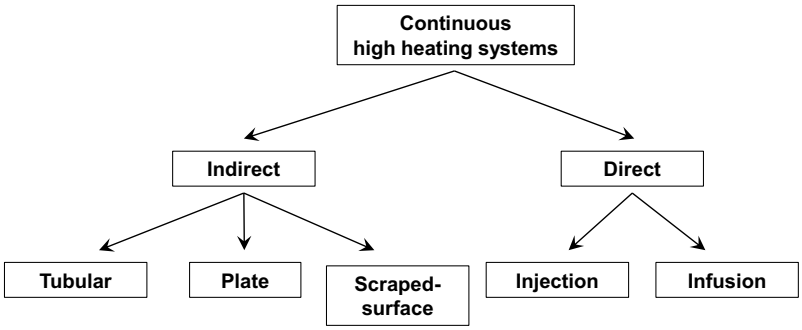

Fig. 1-9: Different types of continuous high heating systems using hot water or saturated steam as a heat transfer medium.

The heat transfer to the product can be achieved by 'indirect' transfer from a heat transfer medium, usually water or saturated steam, through a wall to the product. Heat exchanges for liquid food products can be either designed as concentric tubular heat exchanges, plate heat exchangers or scraped surface heat exchangers for processing of viscous products. Compared to in-container sterilisation, continuous high heating processes have a number of advantages like heat regeneration, rapid heating to high temperature and short holding times, and therefore lower running costs and higher product quality. A concise description of these systems including construction and characteristics, mode of operation, performance and capabilities, advantages and limitations can be found elsewhere (Burton 1994; de Jong 2008).

'Direct' heat transfer is achieved by direct contact of steam with the product. This can be achieved by injecting steam into the product or by distribution of the product into a vessel pressurised with saturated steam. The former is characterized by 'steam-into-product' and is called 'steam injection' and the latter 'product-into-steam' system is called 'steam infusion' as shown in Fig. 1-10 (A) and Fig. 1-10 (B), respectively. The operation principle is the same for both systems except that an additional vessel, the 'infusor' is needed in the case of steam infusion heating. The product is preheated by regenerative indirect heat transfer from heated product for heat recovery. Additional indirect heating by non-regenerative means is required to control the inlet temperature before steam injection or infusion. In this section, the product is pressurised by a restrictor to avoid backflow of steam from the injector or infusor to maintain a constant flow.

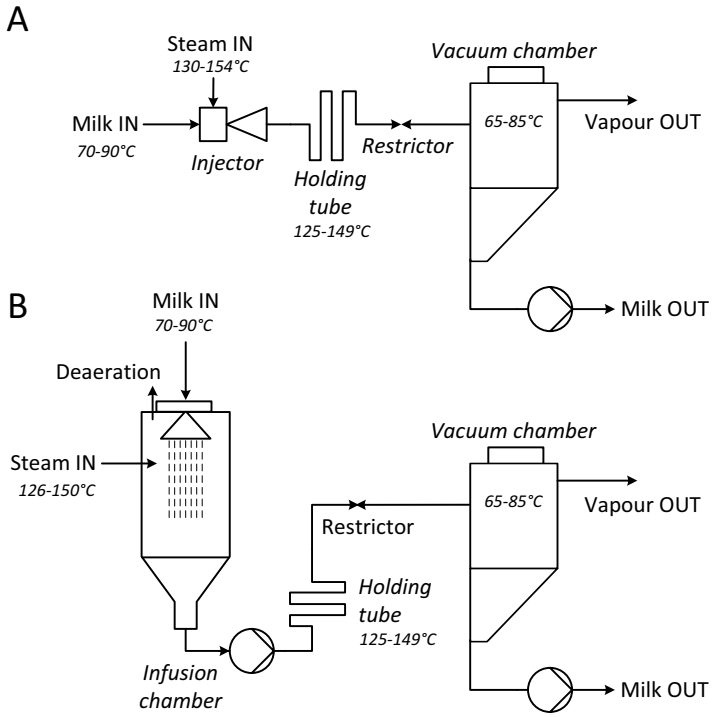

Fig. 1-10: Principle of operation of direct heating systems; (A) direct steam injection, (B) direct steam infusion (Burton 1994).

The contact of the product with saturated steam in the injector or infusor results in very rapid heat transfer. The final product temperature is controlled by the steam pressure supplied. The amount of steam, and therefore of condensate formed, depends on the temperature difference between the inlet and the desired outlet temperature of the injector, the heat capacity of the product, and the condensation enthalpy of steam at the target temperature. The mass flow rate of steam (condensate) is usually in the range of 5-15% of the product flow rate for milk and a resulting temperature difference of 30-75 K.

A higher steam pressure, and correspondingly a higher temperature of the steam are required for steam injection as compared to steam infusion to obtain the same final product temperature. During steam injection heating, a certain proportion of kinetic energy of the steam is needed for intense mixing of steam into the product as small bubbles to give rapid heat transfer. The velocity of the steam in the contact zone with the product is much higher because of the much larger volume of the steam which results in intense mixing and cavitation-like noise within the steam injector. Therefore, steam injectors can be noisy and partial homogenisation of the milk fat can occur depending on the type and geometry of the injector used. Complete

condensation and the final product temperature needs to be achieved at the outlet of the steam injector. A two-phase flow of steam and product after the steam injector would lead to unpredictable fluctuations in heat holding times of the product and therefore variations in terms of microbial inactivation. If complete condensation is achieved, the residence time distribution of the steam injection system and the subsequent holding tube is well defined, especially when some of the kinetic energy of the steam is used to increase the pressure in the holding tube. An example of a commercial steam injector is shown in Fig. 1-11 that comprises three annular gaps to introduce the steam into the annular product flow. The velocity of the product is regulated by an adjustable mandrel. The steam is evenly distributed through bored spacers and condenses into the product at three steam-product contact zones to give a fast condensation by rapid mixing and evenly distributed heat transfer to the product.

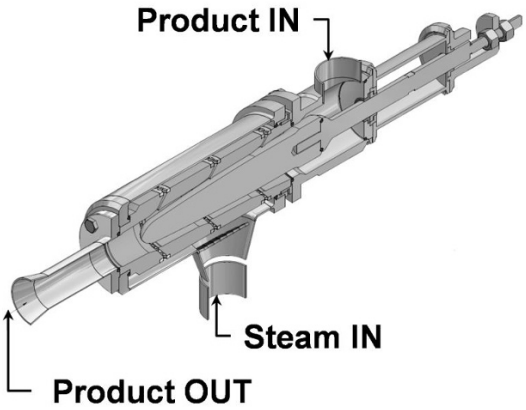

Fig. 1-11: Patented steam injector having three annular steam inlets and an adjustable mandrel to control the velocity of the product flow, bored spacers in stainless steel for the steam inlets, bevelled contact surfaces for the steam inlet into the product, and PEEK material for all product contact surfaces. Noses are forcing higher product velocities which improves the injection effect (Kowalik et al. 2007)[4].

In contrast to steam injection heaters, steam infusion systems distribute the product into the infusor as strings or a (laminar) falling film resulting in the formation of a condensate film on the surface of the product for rapid heat transfer. The product is heated during the passage of the vessel within less than 1 s without any contact to the surface of the vessel. The product temperature at the outlet of the infusor is almost identical to the pressure and temperature of the steam in the vessel. Shear forces are minimal during heating (Schwermann and Schwenzow 2008a). The product is

[4] Patent DE102007017704 A1, Tuchenhagen Dairy Systems GmbH, Sarstedt, Germany. Reproduced with permission by GEA TDS GmbH Sarstedt, Germany © 2016.

collected at the conical jacketed bottom of the vessel that can be 'cooled' to reduce burn-on of the hot product and conveyed further by a pump that compensates for the pressure loss in the holding tube to avoid boiling. A restrictor allows keeping the pressure within the holding tube. A product level in the infusor is critical as it is difficult to maintain constant. In addition, a level in the bottom cone results in difficulties to estimate the residence time distribution and as a consequence, to predict the entire heat holding time of the product. In addition, the accumulation of non-condensable gases within the infusor that interfere with the heat transfer cannot be avoided (Burton 1994). Systems without a level in the infusor were recently developed to overcome these problems (GEA TDS GmbH, personal communication). However, as a consequence, a two phase flow of non-condensable gas and product will be unavoidable.

In both systems, the product passes the restrictor after the holding tube, usually a control valve that keeps the target pressure independent of fluctuations in flow. The decrease in pressure after the restrictor to the pressure level in the expansion vessel (flash cooler, vacuum chamber) results in the formation of vapour. Thereby, rapid cooling of the product to the boiling temperature, corresponding to the vacuum applied, is achieved. The application of vacuum is necessary to reduce the boiling point to a temperature, usually 65-85 °C, at which heat-induced changes are minimal during the short passage to further indirect cooling and to remove the amount of condensate formed after steam injection or infusion. The necessary outlet temperature of the expansion vessel is, in theory, 2 K higher than the inlet temperature at the injector or infusor to avoid concentration or dilution of the product (Burton 1994). However, the outlet temperature is usually lower in practice due to condensate droplets in the saturated steam applied for heating. In addition, the temperature difference between the injector/infusor inlet and the expansion vessel outlet necessary to keep the water balance depends on the size of the system that determines the heat loss to the surrounding. To maintain the vacuum, the vapour is condensed on a surface condenser or injection cooler and the non-condensable gas is removed by a vacuum pump. A centrifugal pump increases the pressure after the expansion cooling and conveys the product to the aseptic homogeniser if required. After homogenisation, the product is cooled against the incoming cold product to filling temperature to recover the residual heat.

The higher sensory quality of directly heated milk products originates from two characteristics of these systems. The condensation of steam on the product surface results in the transfer of the latent heat of vaporisation to the product and come-up times to the final temperature of < 1 s. Compared to indirect heating rates of 1-5 K s^{-1} and therefore come-up times of 25-130 s in indirect systems, far less heat-induced chemical changes in the direct heated product can be achieved. Better sensory properties are mostly a result of the removal of volatile components in the evaporation step (Burton 1994; de Jong 2008). Examples for heating profiles of an indirect heat treatment and a temperature-time profile that can be achieved either by steam injection or infusion are shown in Fig. 1-12.

Direct heating systems were originally designed for continuous sterilisation of liquid milk products, especially when thermo-resistant spores where to be inactivated at very high temperatures. More recently, steam infusion and steam injection heat treatment of milk is also frequently used for the production of ESL milk products due to the superior sensory quality of directly heat treated milk compared to milk processed by indirect heat transfer. However, the lower heat recovery of direct heating systems of 40-50% as compared to up to 90% achieved by indirect heating systems limits the applicability of direct steam injection or infusion mostly to high value products or products with a very high fouling potential due to higher investment and operation costs. The heat treatment of concentrated milk that is such a high value product of high fouling potential was therefore seen to be promising in terms of its sensory quality, heat-induced changes that are increased by an increase in solids concentration, and a maximum of microbial inactivation.

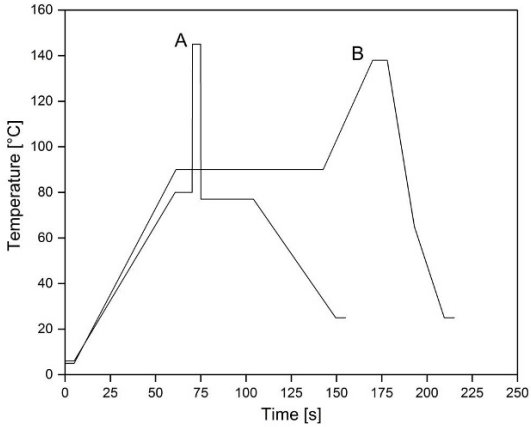

Fig. 1-12: Exemplary temperature-time profiles of direct (A) and indirect (B) UHT plants.

Exclusively indirect high treatment of milk is used to stabilise unconcentrated milk before evaporation and subsequent in-container or UHT-sterilisation in the context of evaporated milk manufacture. Direct heat treatment of the concentrated milk to obtain 'white' condensed milk has not been established to date due to the expected shorter shelf life caused by a higher enzymatic activity and consumer expectations concerning the 'typical' characteristics of evaporated milk (Kiesner et al. 1995). The effect of indirect preheat treatment on the heat stability of concentrated milk will be discussed below.

1.3 Methods for the determination of the heat stability

The origins in the scientific literature on the determination of the heat stability of milk and concentrated milk dates back to the early 20th century. Sommer and Hart (1919) investigated the relationship between titrable acidity, pH, natural variation in milk solids and the heat stability of milk. This was done with the focus on the quality of raw milk for 'condenseries', i.e. manufacturers of evaporated milk. The heat stability was intended to simulate the sterilisation process in an autoclave. This should help to identify heat unstable milk batches that would coagulate during heat treatment of the evaporated milk produced thereof and thereby become inacceptable to the consumer.

Heat stability was determined by heating raw milk in small sealed glass tubes in a xylene vapour bath at 136 °C for rapid heat transfer. The glass tubes were placed in a rack and tilted from time to time until visually detectable coagulation occurred. The entire timespan was then recorded as the coagulation time as samples were heated in the xylene vapour within a negligible timespan of less than 1 min. The aim of this study was to predict the heat stability of the concentrate from raw unconcentrated milk depending on chemical parameters. Sommer and Hart (1919) found that titrable acidity and the pH of raw milk varied so widely that it could not be related to head-induced coagulation. Variabilities in raw milk quality were found responsible for severe coagulation or gel formation during sterilisation of the concentrated milk. They suggested that

- casein required an optimum amount of calcium for maximum heat stability which is in equilibrium with magnesium, citrates, and phosphates present,
- acid fermentation reduced the heat stability of the concentrate due to a decrease in pH, and
- natural variations in the amount of single milk constituents were partly responsible for variations in heat stability.

These statements were taken as a basis for the vast amount of later studies which have, in some cases, completely doubted these statements. However, we should keep these basic statements of Sommer and Hart (1919, 1922) in mind. Water, calcium and magnesium as divalent cations, the anions citrate, phosphate, and pH, i.e. the main constituents of the mineral equilibrium in milk, will be considered as the major factors affecting the casein micelles' colloidal stability in heated concentrated milk as the basic hypothesis.

1.3.1 Subjective heat stability test methods

The test method of Sommer and Hart (1919) was the archetype of all so-called 'subjective' heat stability tests that were developed later on. The subjectivity of the test method refers to the discrimination between the uncoagulated and the coagulated state of a heated milk or concentrated milk sample by visual observation. The principle of all developed test methods remained the same whereby details were varied to

result in a better detectability of the coagulation point to increase precision of the test. In principle, a small amount of sample is filled into a stoppered or screw capped air tight glass tube and immersed in a heat transfer medium. This tube is then tilted, rocked or shaken to avoid burning of the milk onto the glass and proper mixing to transfer and evenly distribute the heat. The experimental set-up developed by Davies and White (1966) is shown in Fig. 1-13 as it became the basis of official standard methods. The timespan after the immersion of the sample into the oil bath and the onset of the formation of visible protein particles ('flocs') is called 'heat coagulation time'. The length of the heat coagulation time is then a (relative) measure for 'heat stability'.

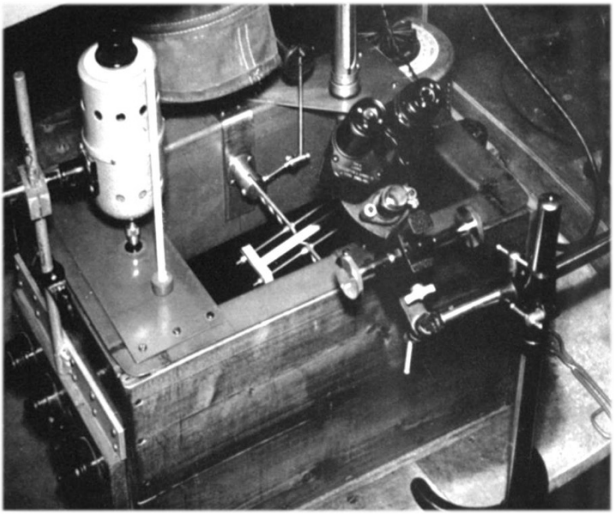

Fig. 1-13: Copper plated thermostatic paraffin bath used for the subjective heat stability test of Davies and White (1966). The sample tube attached to the carriage and the eccentric rocking the tube, a microscope (magnification 8.4), the immersion heaters (left), the stirrer and the thermometer indicating the paraffin bath temperature (left), and the thermometer for regulating the temperature (rear right-hand corner) are shown. The lamp illuminating the sample tube is not shown.[5]

The basic method developed by Sommer and Hart (1919) was later on modified, refined, and standardised by Davies and White (1966) to become a national standard method for heat stability testing. Detailed descriptions of the different methods can be found in the studies of Holm et al. (1932), Cole and Tarassuk (1946), Pyne and McHenry (1955), Rose and Tessier (1959), Belec and Jenness (1962), Davies and White (1966), Farrell and Allgood (1970), and Kneifel et al. (1987). The test method developed by Davies and White (1966) became the so-called 'An Board Bainne' method

[5] Reproduced with permission from Cambridge University Press Ltd, UK © 1966.
https://www.cambridge.org/core/journals/journal-of-dairy-research

which is the national standard method for heat stability testing suggested by the Irish Dairy Board. The Australian standard is also based on the method of White and Davies (1966) and was developed to determine the heat stability of reconstituted concentrated milk at 20% non-fat total solids. Variations in heat coagulation time can be attributed to differences in the test setup and procedure. The different methods were also tested for comparability and it was found that the heat coagulation time of the same milk sample tested varied widely. However, the coagulation times of different samples each tested with two different heat stability test methods gave a positive correlation (Kneifel et al. 1987).

The major difference between these two methods is that the sample volume used is 2.5 mL in the Irish method and 30 mL in the Australian standard method which results in different heating up times. In addition, the Australian standard method is a semi-continuous method. This means that samples are removed from the oil bath every 3 min to detect the onset of coagulation and placed back until coagulation was detected. These two differences account for the much longer coagulation times observed by this method when compared to the Irish Dairy Board method (Foissy and Kneifel 1984; Kneifel et al. 1987).

Differences within individual test methods refer to the

- medium for heat transfer (mineral or silicone oil, a metal block, vapour),
- amount of sample filled into the tube and the size of the tube,
- mode of movement of the tubes (tilting, rocking, shaking or none),
- procedure (one single tube or multiple tubes immersed at different times),
- visual detection of coagulation at the glass surface or after removal from the glass in a petri dish, and
- the measurement temperature (110-140 °C).

The most important aspect of the standardisation process to exclude unknown variables was the choice of the temperature of the heat transfer medium, i.e. the maximum sample temperature. It was fixed to 140 °C for unconcentrated milk. The measurement temperature of 120 °C for concentrated milk containing 18-21% non-fat total solids was used as the standard for evaporated milk. The temperature dependency of the coagulation process was only investigated in early studies on heat coagulation of milk.

Therefore, we have to state that these standard methods do neither give information about the kinetics of the coagulation process nor about the temperature dependency. The coagulation times of samples that coagulate already during the come-up time to > 120 °C cannot be appropriately determined although such severe heat treatments might not be necessary for industrial heat treatments in many cases. In addition, dissociated material from casein micelles which is not visible to the eye was not referred to as heat-induced destabilisation of casein micelles as it does not contribute to visible coagulation. This might be one of the major limitations of this test method as it only defines heat stability of casein micelles as the tendency to form heat-induced protein aggregates.

1.3.2 Objective determination of heat-induced coagulation

The objective methods to determine the heat stability can be divided into two categories. We can define the first category as automated methods that detect the onset of coagulation. These methods were used to replace the subjective decision of the onset of coagulation by physical parameters that are automatically measureable. The onset of coagulation of a milk sample was determined, e.g. by changes in viscosity or the change in the ultrasonic velocity or attenuation. Changes in viscosity due to gel or particle formation at the onset of coagulation were monitored by a heated falling ball viscosimeter, the so called 'Klaro-graph' developed by de Wit et al. (1986), viscosity measurements of the concentrate after sterilisation (Kieseker et al. 1984; Kieseker and Aitken 1988) or by an iron piston that is continuously moved by a ring magnet through the heated milk (Foissy and Kneifel 1984; Kneifel et al. 1987). The use of high resolution ultrasound also allowed following the coagulation process to some extent. However, only the onset of coagulation was extracted from the data and compared to the visual determination (Lehmann and Buckin 2005). All the automated methods resulted in comparable results to the subjective method. Despite these advances, the subjective heat stability testing procedure remained the standard, possibly due to the ease of operation of the method.

The second category is comprised of methods that follow the process of heat-induced coagulation over time. The major disadvantage of these methods is the higher analytical effort needed due to many samples that need to be prepared, heated for different times, and subsequent separation and analysis. The separation of the aggregates is performed by filtration or centrifugation. The pore size of the filters and the centrifugation conditions chosen were of empirical nature in all cases as a quantitative separation of aggregates could not be ensured by, e.g. particle size measurements. The residual protein in the filtrate or supernatant was then determined and taken as a measure for the extent of heat-induced coagulation (Whitney et al. 1952; White and Davies 1966; White and Sweetsur 1977; Nieuwenhuijse et al. 1991). These methods were not applied to a large extent as the course of coagulation was mostly found to be irrelevant for practical applications. The information considered as relevant was the time of sample stability from the beginning of the heat treatment to the onset of coagulation. Graphs as described by White and Davies (1966) are shown in Fig. 1-14. The graphs of coagulation protein can be divided into three phases:

- The 'induction period' or colloidal stability where no coagulation is detectable,
- aggregation of proteins where the coagulum is formed, and
- the stationary phase, where no further coagulation is detectable.

The relative amount of coagulable protein over time also points out that it is the casein that coagulates. The amount of non-coagulable proteins was used to differentiate 'poor coagulation' from 'good coagulation'(White and Davies 1966). Despite these colloquial expressions, coagulation is regarded as an undesired reaction in milk in the context of high heat treatment of liquid milk products.

The additional information about the course of coagulation and the upper asymptote of the graph as compared to the subjective heat stability test, which indicates

only the onset of coagulation (vertical dashed line), was used for a modelling approach. White and Sweetsur (1977) modelled the kinetics of the coagulation of unconcentrated using a formal first order reaction kinetics approach. They suggested that a kinetic description of the coagulation process was possible when the 'induction period' and the stationary phase is excluded from the calculations. White and Sweetsur (1977) could derive an activation energy E_A, rate constants k, and a temperature factor (Q_{10}) using this formal reaction kinetic approach. However, they found that the residuals, i.e. the difference between the experimental data and graphs predicted by the model were not evenly distributed over heating time. Considering the omission of (precise) measurement data, this indicates that the model used could be improved. The extension of the model would be possible by the introduction of parameters that consider the induction phase and the stationary phase. Though, the non-normal distribution of residuals indicates that another model might be necessary to describe the data.

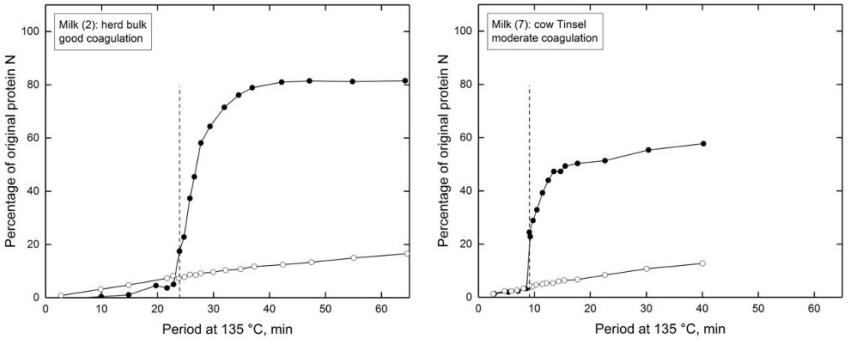

Fig. 1-14: Aggregated protein in percent (●) and non-protein nitrogen (NPN) plus proteose peptone nitrogen (PPN) (○) produced in 'normal' separated bulk milk and milk from an individual cow after heating at 135 °C for various periods. The vertical broken line indicates the visible onset of coagulation (White and Davies 1966). Data were extracted using the digitiser of OriginPro 2017G.

The dependency of the course of coagulation of concentrated milk depending on pH was investigated by Nieuwenhuijse et al. (1991). They found that about half of the protein in concentrated milk was coagulable independent of pH. However, no kinetic modelling approach was applied to describe the course of the coagulation process. However, they confirmed that the onset of coagulation determined by the objective test method could be readily detected by the subjective test method whereby the information on kinetics of the coagulation process is lost using the subjective test method.

1.4 Factors that affect heat stability of milk and concentrated milk

The considerable amount of research on the heat stability of unconcentrated and concentrated milk over the last 100 years had basically two objectives, one being more based on fundamental research and the other driven by the need to advance dairy processing and product quality, e.g.

- the chemistry and physics of heat-induced changes on caseins and casein micelles that lead to destabilisation of the colloidal state and subsequent aggregation,
- compositional aspects and processing factors such as technological treatments and additives that affect the heat and storage stability of commercial milk and concentrated milk.

Using the described subjective heat stability method of White and Davies (1966) and to a much lesser extent any kind of objective heat stability test method, it was possible to identify the relevant factors that affect the heat stability of milk and concentrated milk. Due to the high heat stability of unconcentrated milk, research was focused on the former aspect. Due to the conflict between the low heat stability of concentrated milk and the destabilising effect of the (UHT-)sterilisation process, research on concentrated milk was often focussed on the control or improvement of the heat stability of concentrated milk by using (permitted) additives and technological treatments of milk or combinations thereof.

Walstra et al. (1984) stated that only few subjects in dairy chemistry have been studied more intensively than heat stability of milk and concentrated milk and are less understood. Excessive literature has been added, especially in the last 40 years, further elucidating the factors affecting the heat stability of milk and concentrated milk. Further proposals on possible mechanisms involved have been given. A conclusive theory of heat-induced coagulation, of the mechanisms and the kinetics that integrates all confirmed observations, however, is still lacking.

This virtually provokes further research in this direction as the practical relevance of the heat stability of dairy concentrates is further increasing with increased milk production, increased awareness of microbial hazards, and therefore intensified thermal processing. Practical means and guidelines have been developed for evaporated milk to avoid heat-induced coagulation (Muir 1984). The factors, i.e. compositional aspects and processing, that affect heat stability the most are well known without, however, a full understanding of the processes involved (Singh and Creamer 1992). A selection of current knowledge on these factors will be presented with special reference to the scientific insights that fostered the results of this thesis.

The factors that affect the heat coagulation time as determined by the subjective test method can be categorised into three main categories:

- The heat stability testing procedure, mainly the heating temperature,
- the intrinsic heat stability of milk and concentrated milk, mainly dependent on milk serum composition, and
- modification and processing of milk before the test that affect the colloidal stability of the casein micelles.

1.4.1 Dependency of stability on pH and heating temperature

Rose (1961a, 1961b) first described the marked effect of initial milk pH on heat stability of milk in the range of 6.2-7.4 after adjustment by acid or based addition. He defined two types of milk that differed in their heat coagulation time (HCT)-pH profile as shown in Fig. 1-15. Type A milk shows a maximum in HCT usually at the natural pH of milk at pH 6.6-6.8 and a local minimum at pH 6.9-7.0. Type B milk shows an increase in HCT with increasing pH. Most of the milk from individual cows and pooled bulk milk show the behaviour of type A milk. Removal of serum proteins from type A milk results in milk that shows a pH-dependency similar to type B milk. Partial removal of salts from milk by dialysis against deionised water also leads to a conversion of a type A to a type B profile. These observations point out the implication of milk salts and whey proteins in the coagulum formation at pH > 6.7. A low heat stability of both milks at low pH was attributed to 'salt-induced' coagulation. This means that the colloidal stability of casein micelles was assumed to be lowered by a decrease in surface charge of casein micelles, a reduced electrostatic repulsion, and a collapse of the hairy layer due to charge neutralisation. This results in an increased tendency towards aggregation (van Boekel et al. 1989a). An increase in calcium activity and ionic strength by the dissolution of CCP could also contribute to an increase in attractive forces between casein micelles (Nieuwenhuijse et al. 1991). At pH < 6.7, denatured whey proteins adsorbed to the micelle surface will increase the heat stability compared to serum protein-free casein micelle dispersions by the formation of a less calcium sensitive steric barrier.

The heat stability of concentrated milk at 20% non-fat total solids is low compared to unconcentrated milk. At pH > 6.7, the rate of the dissociation of heat-induced whey protein-κ-casein complexes is increased. This was correlated to a destabilisation of caseins micelles for both, concentrated and unconcentrated milk (Singh 2004). When the same measurement temperature is used, the HCT of concentrated milk at 20 % total solids is approximately 25 % of that of unconcentrated milk at the pH maximum as shown in Fig. 1-15, indicating a relationship between the total solids content of the concentrate and the HCT.

These observations point out that heat-induced coagulation is a complex, at least two-stage process of destabilisation and aggregation that is affected by multiple milieu conditions which mutually influence each other. However, it is still unclear which reaction is rate determining. At different pH, different reactions like chemical, e.g. cleavage of covalent bonds or cross-linking, or physical reactions, e.g. dissociation of κ-casein might be rate determining (Walstra et al. 1984). However, it remains uncertain if all physico-chemical reactions taking place during heat treatment that affect casein micelle colloidal stability have been identified.

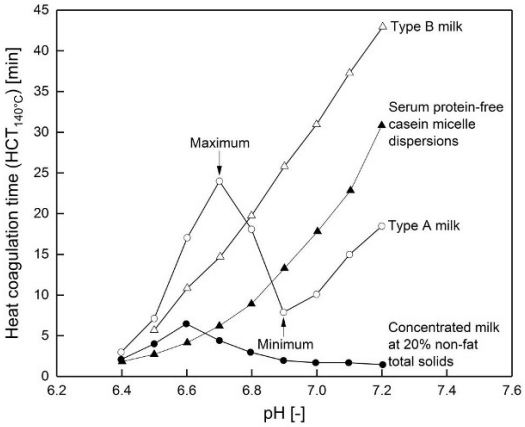

Fig. 1-15: Heat coagulation time (HCT) vs. pH profile for type A milk, type B milk, concentrated milk at 20% non-fat total solids, and serum protein-free casein micelle dispersions heated at 140 °C (Singh 2004). Data were extracted using the digitiser of OriginPro 2017G.

Besides compositional aspects, early studies on the heat stability of milk also investigated the effect of the heating temperature on heat-induced coagulation of unconcentrated milk. This was done to optimise measurement parameters of the subjective heat stability test and to investigate the kinetics of the onset of coagulation as shown in Fig. 1-16.

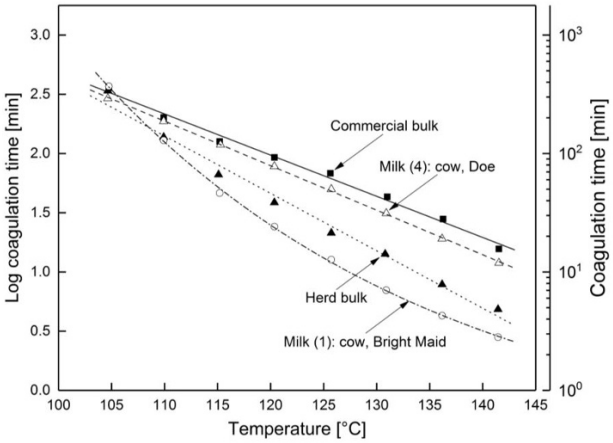

Fig. 1-16: The relation between the logarithm of coagulation time and temperature of four different types of milk (Davies and White 1966). Data were extracted using the digitiser of OriginPro 2017G.

It was found by Davies and White (1966) that the coagulation time decreases with increasing oil bath temperature. The visibility of the coagulum at the onset of coagulation deteriorated as the size of the clots decreased with decreasing temperature. This shows that the rate constant of heat-induced coagulation is considerably accelerated by an increase in temperature. The *z*-value, i.e. the increase in temperature to reduce the coagulation time to 10% as compared to the reference temperature, can be directly derived from the slope of the graphs shown here. The approximate *z*-values for the coagulation time varied between 15 and 27 K for milk of individual cows (Bright maid, milk 1) if linearized and commercial bulk milk, respectively. However, Davies and White (1966) rather assumed a curvilinear behaviour for milk (1), i.e. a progressive increase in the temperature dependency with decreasing temperature. The herd bulk milk of Davies and White (1966) showed a *z*-value of 19 K. This shows that compositional variations will not only affect the coagulation time at the measurement temperature chosen but also the temperature sensitivity of the reaction, i.e. the slope of the regression line.

Therefore, the simplification of the heat stability test method by measurement at constant temperature might also lead to a misinterpretation of the relative comparison of coagulation times. A difference between samples will be visible at higher measurement temperatures that will be invisible at lower measurement temperatures as the temperature sensitivity will be affected by changes in the serum composition (pH, ionic strength, and soluble salts). A known example is the conversion of a type A milk to a type B milk by a decrease in measurement temperature, where the minimum at pH 6.9 simply disappears by decreasing the measurement temperature according to Singh (2004)

We can conclude that the determination of the coagulation point of milk or concentrated milk at a fixed measurement temperature results in a significant loss of information concerning the kinetics and the course of heat-induced coagulation. In addition, the relationship between heating temperature and coagulation time for concentrated milk has not been established to our knowledge. The consideration of the temperature dependency of the HCT and the course of coagulation could lead to a far better quantitative description of heat stability and the heat-induced coagulation process in milk and concentrated milk. A concept of variable or adopted heating temperature during heat stability testing could give a better discrimination of samples with low heat stability. This kinetic description is of outmost importance and there is no way getting around it when we consider the continuous heat treatment of concentrated milk at high temperature for few seconds. As these processes cannot be simulated by the simple heat coagulation test giving coagulation times in minutes at lower temperature, a kinetic model to calculation the effects of heat treatment on concentrated milk in comparison to microbial inactivation is needed.

1.4.2 The effects of processing factors and additives

Processing of concentrated milk to the final shelf stable evaporated milk products includes multiple processing steps that can be arranged in different order and will thereby affect the stability of the concentrated milk towards the final heat treatment, mostly a sterilisation process. The heat stability of concentrated milks is 'low' and seasonal variations in milk composition result in considerable differences in the heat stability. This seasonal variation is especially pronounced in milk production regions with pasture based milk production (Holt et al. 1978a). 'Low' heat stability means that milk concentrated to 18-21% non-fat total solids was found to coagulate during sterilisation even after preheating of the milk depending on season (Singh and Tokley 1990; Pouliot and Boulet 1991). This indicates that a dry matter content of 18-21% of evaporated milk at the fixed heating intensity of the sterilisation process represents a borderline case in terms of the conflict between microbial inactivation and casein micelle stability towards heat. At higher total solids content, all concentrates will be unstable towards (in-container) sterilisation even after preheating of milk and stabiliser addition (Walstra et al. 1984).

Therefore, technological means including processing steps, their order, and the use of additives were often directed towards a control or an 'improvement' of the heat stability of concentrated milk to achieve sterilisation of evaporated milk without coagulation of caseins and subsequent gelation or sediment formation. Processing alternatives for the manufacture of evaporated milk are shown in Fig. 1-17.

The processing of UHT sterilised evaporated milk consists of four main processing steps besides aseptic packaging of the final product which can be arranged in different order. The usual processes are the variants (A) and (B) as aseptic concentration of milk by evaporation or RO have not yet become state of the art and are therefore rather of theoretical relevance.

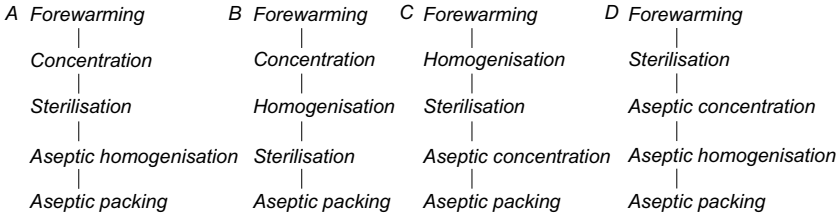

Fig. 1-17: Processing alternatives for the manufacture of evaporated milk or concentrated sterile milk in general using the UHT process for sterilisation. The term 'forewarming' is equivalent to preheating of unconcentrated milk before concentration (Muir 1984).

Preheating of milk

'Forewarming' or 'preheating' of unconcentrated milk was found to considerably increase the heat stability of concentrated milk and is an integral part of concentrated

milk processing before in-container sterilisation. The increase in heat stability by preheating can be predicted by the subjective heat stability test as shown in Fig. 1-18. Heat stability was found to be sufficient for in-container sterilisation (usually 115-120 °C/12-20 min) at the optimum pH after preheat treatment of unconcentrated milk.

Preheating conditions of milk vary widely and were often directed towards a complete denaturation of whey proteins. The reduction of the amount of reactive whey proteins that could crosslink casein micelles in concentrated milk and the coverage of casein micelles with denatured whey proteins were assumed to reduce the rate of heat-induced aggregation during sterilisation at pH < 6.7 (Tessier and Rose 1964). Preheating of milk also results in a shift of the maximum of the HCT-pH profile to the 'acid' side (pH < 6.6) (Rose 1962). Due to the seasonal, i.e. compositional variation in the HCT-pH profile, the effectiveness of the preheat treatment varies (Singh and Tokley 1990). The main effect of the preheat treatment is, however, that the natural pH of concentrated milk comes closer to the pH of maximum stability and thereby heat stability increases without adjustment of the pH in the concentrate. Usual preheating conditions for milk used are e.g., 90 °C/10 min, 110-120 °C/1-5 min, 140 °C/5 s or 130-150 °C/1-5 min (Webb et al. 1943; Newstead et al. 1979; Sweetsur and Muir 1982; Singh and Tokley 1990; Fox et al. 2015). Concentration of milk by thermal evaporation at 55-85 °C might also lead to a shift in the heat stability maximum to a more acidic pH due to possible heat-induced changes in milk depending on residence time and temperatures of the milk in the evaporator.

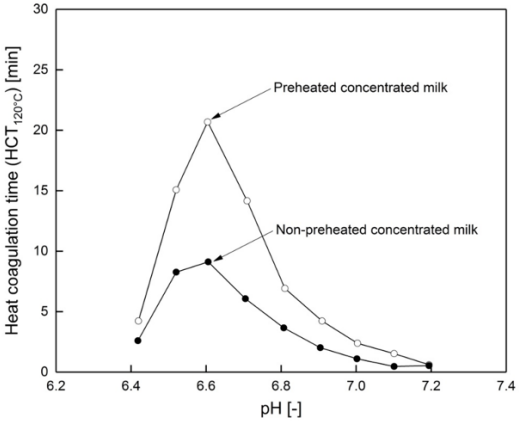

Fig. 1-18: HCT vs. pH profile for concentrated milk at 20% non-fat total solids prepared from either preheated or non-preheated milk heated at 120 °C. Preheating conditions were not indicated (Singh and Creamer 1992). Data were extracted using the digitiser of OriginPro 2017G.

All these preheating conditions were tested in the context of the subjective heat stability test and in-container sterilisation. Very little is known about the effectiveness of preheating in terms of an increase in the heat stability of heated concentrated milk

towards continuous UHT heat treatment. Quantitative data for the increased temperature-time combinations possible without coagulation of concentrated milk depending on total solids content are unknown. Processing conditions for continuous sterilisation therefore remain largely of empirical nature.

Concentration of milk

After preheating, milk is usually concentrated by evaporation or RO. Little quantitative data exist on the decrease in heat coagulation time with increasing heating temperature and total solids content for both, concentrates from preheated and non-preheated milk. However, it was stated by Walstra et al. (1984) that milk concentrated to > 40% total solids even coagulates at temperatures of < 100 °C which results in gel formation that rather resembles the phenomenon of age-gelation as observed for concentrates when stored at ambient conditions. To date, little correlation could be found between the heat stability of unconcentrated milk and concentrated milk prepared thereof by evaporation (Walstra et al. 1984; Singh 2004). However, when we argue that the only difference in composition of milk and concentrated milk is the water content, there should be a detectable correlation. An incremental decrease in water content should result in an incremental decrease in heat stability. The evidence for a correlation might be hampered by the fact that the HCT measurement temperature for concentrated milk is usually different from unconcentrated milk. This impairs comparability of the results as discussed in the previous section. In addition, the evaporation process at elevated temperature affects the pH-sensitivity of concentrated milk compared to unconcentrated milk which might result in the denaturation of whey proteins and changes in the milk salt equilibrium during evaporation. Cold concentration of milk by RO might be a technique to avoid these changes.

Concentration of milk by porous membranes such as ultra- or microfiltration results in higher heat stability of the milk protein concentrate when the same factor of concentration is compared to RO concentrated milk or milk concentrated by evaporation. This is due to several reasons, e.g.

- a reduced concentration of soluble salts, especially soluble calcium as the serum composition of milk concentrated by porous membranes is similar to unconcentrated milk,
- a pH that is closer to the pH of the maximum stability of milk, and
- a reduced amount of whey proteins when microfiltration is applied which especially increases the heat stability at pH > 6.7 and results in a HCT-pH profile of type B milk.

Diafiltration of milk using deionised water further increased the heat coagulation time of the final concentrate possibly due to an increase in pH, reduced ionic strength, a further removal of whey proteins (Sweetsur and Muir 1980; Muir and Sweetsur 1984). However, later studies found that demineralisation of milk by excessive diafiltration can lead to very poor heat stability of milk protein concentrates (Crowley et al. 2014; 2015). Diafiltration media that maintain the serum composition and pH of milk like simulated milk ultrafiltrate (SMUF) without lactose could lead to

optimal heat stability of the concentrate and further insights into heat-induced changes in whey protein-free milk systems with modified serum composition.

Milk fat and homogenisation

Milk fat or lipid droplets *per se* in the unhomogenised state have little effect on the heat stability of milk indicated by the coagulation times of unconcentrated skim milk and whole milk which were found to be similar (Sweetsur and Muir 1983a). Homogenisation of skim milk in the usual range for homogenisation of milk (≤ 35 MPa) had little effect on the heat stability of concentrated skim milk (CSM) (Sweetsur and Muir 1983a). Much higher pressures as used for ultra-high-pressure homogenisation (> 180 MPa), e.g. for microbial inactivation, are required to affect the heat stability of skim milk (Sandra and Dalgleish 2005). From these observations, we can conclude that the interaction of casein micelles with fat globules at the oil-water interface results in structural rearrangements that render the casein micelles instable towards heat. Homogenisation of milk entails the disruption of fat globules from around 2-10 μm to a size less than 2 μm and the subsequent adsorption of milk proteins to form a secondary fat globule membrane on the newly formed oil/water interface. This secondary layer inhibits the aggregation, coalescence, and creaming of fat globules during storage. Aseptic homogenisation, i.e. homogenisation after the final heat treatment of concentrated milk containing milk fat, is usually preferred for evaporated milk manufacture by the UHT process. This is due to the fact that homogenisation of milk prior to concentrating or homogenisation of the concentrate results in a significant decrease in heat stability of the final concentrates. It was suggested that the secondary fat globule membrane of caseins and whey proteins on the surface of the newly formed fat droplets was responsible for a reduction in heat stability. The destabilising effect of homogenisation increases both with increasing temperature and homogenisation pressure, i.e. with increasing fat globule surface area (Sweetsur and Muir 1983b; Muir 1984; McCrae et al. 1994; Hinrichs et al. 1998).

When in-container sterilisation of the concentrated milk is used for preservation, homogenisation needs to be performed before sterilisation. This leads to a conflict between the creaming of the concentrate during storage and the heat stability of concentrate. When low homogenisation pressures are used to keep the concentrate sufficiently heat stable, creaming and cream plug formation will occur within the containers. By using UHT heat treatment in combination with aseptic homogenisation, this conflict can be avoided as homogenisation is performed after UHT sterilisation.

Addition of additives to influence the heat stability of concentrated milk

Practical means to improve the heat stability of concentrated milk focus on the main components involved in heat-induced coagulation. Permitted additives were used in industrial practice. In research, other chemicals were also used for targeted investigations on the mechanisms of heat-induced coagulation.

Permitted additives to enhance heat stability of concentrated milk comprise

- changes on the charge of casein micelles by adjustment of the pH by NaOH, HCl, (alkaline or acid) phosphates and citrates of sodium and potassium,

- the complexation of excess soluble calcium present due to the removal of water during concentration by complexing agents (phosphates, citrates, EDTA) (Pouliot and Boulet 1991; de Kort et al. 2012),
- addition of phospholipids as pure chemicals or from natural sources such as buttermilk (Singh and Tokley 1990).

Targeted modification of milk constituents in research was performed to elucidate components involved in coagulation and the mechanisms of heat-induced coagulation using

- oxidizing agents that modify the charge of amino acid residues (H_2O_2, $KBrO_4$, KIO_3) (Singh and Fox 1987b),
- the blockage of thiol-disulphide exchange reactions (N-ethyl maleimide) or reducing agents such as β-mercaptoethanol, dithiothreitol or cysteine that will reduce inter- and intramolecular disulphide bridges (Singh and Fox 1987b),
- the reduction of the dissociation of caseins from casein micelles, mainly κ-casein by enzymatic (transglutaminase) (Huppertz 2014), thermal or chemical crosslinking (formaldehyde and various dialdehydes) (Holt et al. 1978b),
- addition of κ-casein or hydrolysis of κ-casein by chymosin (Fox and Hearn 1978c),
- addition of ethanol or other alcohols to milk that markedly reduces its heat stability (Horne and Muir 1990; Horne 1992).

Despite the effects of additives on the heat stability of concentrated milk, none of the described additives, not even unapproved chemicals for food use were able to restore the original heat stability of unconcentrated milk. Hence, in a first attempt, a quantitative description of the heat stability in terms of heating temperatures and times without noticeable coagulation for continuous sterilisation needed to be addressed. The quantitative investigation of the improvement of the heat stability by additives or preheat treatment of the unconcentrated milk would then further advance the continuous thermal processing of concentrated milk.

2 Motivation and objectives

The vast amount of literature on the heat stability of milk and concentrated milk demonstrates the importance of heat stability in dairy research and industry. A brief and comprehensive summary was given in the theoretical background. It was intended to present the historical and recent approaches taken to investigate the heat stability of milk and, most relevant, concentrated milk. The lack of knowledge, especially in terms of the kinetics of heat-induced coagulation of concentrated milk, was pointed out. A quantitative description of the critical temperature-time combinations for the heat treatment of concentrated milk was therefore one of the guiding ideas.

During the studies for this thesis, the research on the kinetics of thermal inactivation of microorganisms, bacterial spores, enzymes, and degradation of nutrients in food science was taken as a role model. Fundamental understanding of the kinetics and parameter estimation enabled major advances in continuous thermal dairy processing. The systematic investigation of the inactivation and degradation processes enabled the mathematical prediction of working ranges to maximise desired effects and to minimise undesired reactions, all in all intended to keep product quality and maximise product safety.

Starting from laboratory scale, the quantitative relationship between the possible heat load in terms of heating temperature and time depending on total solids content of concentrated skim milk (CSM) was investigated. A subjective heat stability test that allows for the comparison of the lab scale experiments with continuous heat treatment of CSM on pilot scale by using kinetic dependencies was developed. The quantitative description of changes in heat stability of CSM by an increase in total solids content, by preheating of milk, and addition of milk fat was intended.

The use of continuous direct steam injection (DSI) heat treatment enabled the quantitative description of the heat stability of CSM on pilot scale. Further investigations on the mechanism of heat-induced coagulation of CSM by analysis of the different size fractions formed during coagulation of CSM by quantitative differential centrifugation were performed. The coagulation process was followed to gain mechanistic insights into the heat-induced coagulation process, the reactions preceding coagulation, and the distribution of caseins during coagulation.

Further investigation of the course of the coagulation of indirectly heat treated CSM over much longer heating times under isothermal conditions at different temperatures were performed. The quantitative data resulted in a mathematical model

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https://doi.org/10.1007/978-3-658-19696-7_2

capable to describe the onset of coagulation, course of coagulation, and the temperature dependency of heat-induced coagulation of CSM at different total solids content. Based on this model, a prediction of the heat stability data obtained using DSI was possible.

Further investigations on the heat stability of casein micelles in general aimed at the selective modification of the milk serum composition and the investigation of the structure of casein micelles in concentrated milk and milk systems. The reason for the decrease in the heat stability of concentrated milk should be further elucidated by using a micellar casein concentrate in simulated milk ultrafiltrate as a model milk system. The effects of pH, soluble calcium, and ionic strength on structure and the colloidal stability of casein micelles were addressed.

The results section of this thesis is structured according to publications relating to the main aspects of research on the heat stability of concentrated milk and the effect of milk serum on the structure of the casein micelle in concentrated milk systems as listed below. Investigations on

- the heat stability of CSM on lab scale,
- the heat stability of CSM on pilot scale using direct steam injection,
- the dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection,
- milk ultrafiltrate analysis by ion chromatography and calcium activity for the preparation of a simulated milk ultrafiltrate,
- modelling of heat stability and heat-induced aggregation of casein micelles in concentrated skim milk using a Weibullian model, and
- the implication of pH and soluble calcium on micelle size and dissociation of κ-casein in relation to heat stability of micellar casein

were conducted.

3 Heat stability of concentrated skim milk on lab scale[6]

Abstract

The destabilising effect of heat treatment on skim milk concentrated by reverse osmosis was shown to be strongly dependent on heating time and temperature. Heat-induced coagulation could be described as a function of heating time and temperature over a broad range of non-fat total solids concentration using the described test method. This improved test method in terms of a more instant and better visibility of coagulation effects can be used for a wide range of total solids. Heat stability strongly decreased as total solids content increased as a function of heating time and temperature. A clear correlation between the heat stability of unconcentrated skim milk and concentrates thereof could be shown. In this case, the heat stability of unconcentrated skim milk can be used to estimate the heat stability of concentrated skim milk as variation in milk composition of pooled bulk milk from a local dairy plant was seen to be low.

6 Original publication: Dumpler J, Kulozik U (2015) Heat stability of concentrated skim milk as a function of heating time and temperature on a laboratory scale - Improved methodology and kinetic relationship. Int Dairy J 49:111–117. doi:10.1016/j.idairyj.2015.05.005. Adapted original manuscript. Adaptions of the manuscript refer to enumeration type, citation style, spelling, notation of units, and format. Permission for the reuse of the article is granted by Elsevier Limited.

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https://doi.org/10.1007/978-3-658-19696-7_3

3.1 Introduction

Various methods have been developed to assess the heat stability of milk and concentrated milk made thereof (Davies and White 1966; Kneifel et al. 1987; Kelly and Roefs 1990; Lehmann and Buckin 2005). Most of these methods are based on heating a small amount of sample in a glass tube in oil or another liquid appropriate for heat transfer at temperatures beyond 115 °C. Oil bath temperatures are chosen depending on the estimated heat stability of the samples between 115 and 140 °C, which introduces some degree of inconsistency due to different heating-up ramps and, thus, different heat coagulation times (HCT). Heat-induced coagulation can be determined visually (Sommer and Hart 1919; Rose 1961a; Davies and White 1966; Farrell and Allgood 1970; Darling 1980), by changes in viscosity as well as ultrasonic spectroscopy using automated methods (Leviton and Pallansch 1961a; Foissy and Kneifel 1984; Lehmann and Buckin 2005) or by changes in filterable or sedimentable nitrogen for endpoint determination (Whitney et al. 1952; White and Davies 1966).

Despite these differences, HCT is usually reported as a result of these test methods mostly without referring to the heating profile included in the test procedure. Methods of this kind may be to some extent useful to simulate batch sterilisation in an autoclave where heating of the sample is as slow as it is in heat stability tests on a laboratory scale. Recent developments in this area of research tried to avoid subjectivity by means of semi-automated methods using physical changes of the sample to determine the induction period of coagulation.

Nevertheless, some of the subjective test methods developed in research have become national standards such as the widely used test of Davies and White (1966). Thus, depending on the apparatus and oil bath temperature used, the HCT for a certain system varies widely and merely gives information about the underlying mechanism as well as temperature-time combination adjustable in a continuous flow heating process. Despite this, HCT of national standard procedures were seen to correlate positively using the same batches of milk for testing (Kneifel et al. 1987; Kelly and Roefs 1990).

Early publications on heat stability of concentrated milk were often dedicated to the development of an appropriate measurement procedure varying also the oil bath temperature. As a result of these method development efforts, it was shown that HCT increased with decreasing oil bath temperature (Webb and Holm 1932; Davies and White 1966). Measurement temperatures of national standards were then set to 140 °C for unconcentrated milk and 120 °C for concentrated milk. Information about a temperature-dependence of heat-induced coagulation therefore was lost. Despite this, major influencing factors on heat stability of concentrated and unconcentrated milk could be unravelled. Especially pH-dependency of HCT as well as the effects of different mineral composition of the serum phase, whey protein denaturation and technological pre-treatments such as various preheat treatments of the milk used and the effect homogenisation were investigated (Newstead et al. 1979; Sweetsur and Muir 1982; McCrae et al. 1994; Whiteley and Muir 1996; Tan-Kintia and Fox 1999).

Nevertheless, the reaction kinetics eventually leading to the formation of large particles should be further addressed.

Most of the research of the last decades on the one hand has focused on heat stability of unconcentrated milk for research purposes. As caseins were shown to withstand up to 20 min at 140 °C, heat stability tests of unconcentrated milk of good quality is not of practical relevance, especially since raw milk quality has generally been improved worldwide. Singh (2004) stated that HCT using unconcentrated milk often correlates very poorly with heat stability of concentrated milk under commercial sterilisation regimes. This may be due to the fact that temperature-time combinations at the transition from the homogeneous state to coagulum formation depend on the heat stability test used as well as on milk composition. Milk composition and hygienic state is much more uniform today as compared to decades ago due to more awareness and hygiene standards as well as similar feeding strategies independent of season in central Europe.

On the other hand, research on concentrated milk was focused on concentrates used for condensed milk manufacture which has a total solids content close to the national standards for evaporated milk including defined amounts of milk fat (Leviton and Pallansch 1961b; Singh and Tokley 1990; Singh 2004; Crowley et al. 2014). Fat contributes to dry matter, but was seen to be of major relevance regarding its impact on heat stability of milk concentrates only in its homogenised state (Sweetsur and Muir 1982; Hinrichs 2000). Heat stability of concentrates exceeding non-fat total solids content of that resulting from a volume reduction by a factor of three has not been published to our knowledge. Heat stability of these concentrates may have been too low for analysis under standard measurement conditions even at 120 °C. However, concentrates of higher total solids content and satisfactory shelf life would be of major interest for the partial replacement of milk powder in applications where milk powders have to be reconstituted to be mixed into liquid dairy products. This may contribute to an increase in resource and cost efficiency in the dairy industry.

The aim of this study was to investigate skim milk concentrates up to 35% total non-fat solids for their heat stability concerning temperature-time relationships of heat-induced coagulation and to establish a modified and standardized HCT test method allowing for a faster heat stability testing within few minutes, the adaption of the subjective test to various total solids as well as minimization of subjectivity and, thus, less variable results.

3.2 Materials an methods

Materials and concentration of skim milk by reverse osmosis (RO)

Pasteurised (74 °C for 28 s) or low pasteurised (68 °C for 28 s) skim milk was obtained from a local dairy factory (Molkerei Weihenstephan, Freising, Germany). The average main composition of the skim milk used (mean ± standard deviation) was 9.55 ± 0.12 % of total solids, 44.9 ± 1.2 g L^{-1} lactose (analysed with high performance

liquid chromatography; HPLC), 1689 mg L^{-1} ± 86.8 potassium, 448 ± 42 mg L^{-1} sodium, 1414 ± 133 mg L^{-1} total calcium (analysed with flame photometry), 5.17 ± 0.88 g L^{-1} major whey proteins α-lactalbumin, β-Lactoglobulin B and A (analysed with reversed phase-HPLC; RP-HPLC) as well as 3.7 ± 0.09 % of total protein (method according to Dumas; ELEMENTAR Vario max cube; Elementar Analysensysteme GmbH, Hanau, Germany).

Pasteurised milk was concentrated by reverse osmosis (RO) on a pilot scale at 9-10 °C at a transmembrane pressure of 45-50 bars up to 36 % of total solids without further storage and preheat treatment often used to increase the heat stability of the concentrates. The membranes used had a salt retention >99.2 % based on standard testing conditions (FILMTEC™ SW30-2540, FilmTec Corporation, Edina, MN, USA and KOCH TFC®-SW 2.5'', KOCH Membrane Systems, Aachen, Germany).

Sample preparation for heat stability testing and pH measurement

Skim milk concentrates of different total solids were prepared by redilution using RO permeate calculating permeate addition by volume reduction (VR) at a certain total solids level in relation to the VR of the RO concentrate obtained. After dilution, samples were analysed for exact total solids content using a microwave dryer (CEM Smart Turbo 5, CEM, Kamp-Lintfort, Germany).

The adjustment of pH in concentrated skim milk for the determination of heat stability time and temperature was done by dropwise addition of 1 N or 0.1 N NaOH or HCl under vigorous stirring. Heat stability measurements were performed in triplicate. Results are reported as mean ± standard deviation.

Determination of heat stability

Heat stability of RO concentrated skim milk was determined by a modified oil bath method. The assembly is shown in Fig. 3-1. Samples were filled into a 50 mL round bottom screw capped borosilicate glass tube (Schott, Zwiesel, Germany) and tightly screwed. The height of the tubes was 98 mm, outer diameter was 34 mm and the screw caps were GL 32.

Samples of 10 mL were used to determine the heat coagulation measurements. A Pt100-2 temperature sensor (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany) was tightly screwed into the screw cap and connected to a ALMEMO® 2590-4S (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany) for data recording. Samples were immersed into the oil bath at a constant temperature of 155 °C in such a way that the level of the sample was equal to the oil bath level to control the slope and final temperature of the heating curve shown in Fig. 3-2. Data of pH and temperature were recorded every 3 s. An oil bath temperature of 155 °C was chosen for standard measurement procedures as a compromise between a high maximum temperature which gives a fast coagulation of the samples and scorching of concentrated skim milk of high total solids onto the surface of the glass tubes.

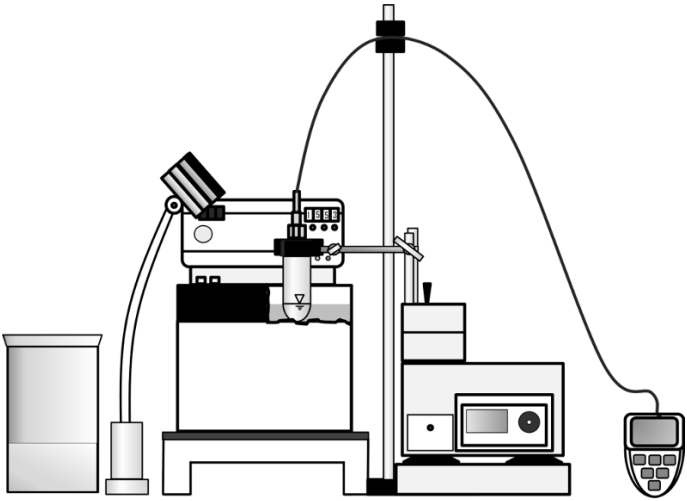

Fig. 3-1: Assembly used for determination of heat stability of unconcentrated and concentrated milk of different total solids content. Testing equipment consists of an ice-water mixture for sample cooling, an oil bath for sample heating, a lamp, a shaker equipped with a support for fixation of the screw capped round bottom borosilicate tubes and an ALMEMO® 2590-4S to record time, temperature and pH during a trial.

Measurements of HCT at different oil bath temperatures were performed by adjustment of the oil bath from 95 up to 165 °C in steps of 10 °C to demonstrate the impact of various maximum temperatures and, therefore, of different heating-up profiles as was the case in the earliest studies on HCT of milk and milk concentrates. The trials were done twice with different lots of skim milk whereby each sample was measured in triplicate. The partial immersion of the glass tubes resulted in a maximum target sample temperature that was 18-20 K below the oil bath temperature. Sample tubes were shaken at 300 min^{-1} inducing centrifugal forces which led to a thin film of concentrated milk on the glass surface above the filling level of the screw capped tubes. Thus, it was made easy to detect coagulation and subjectivity of detection of the appearance of the first aggregates could be minimized. Fouling on the glass tubes due to the high temperature difference was not detected as induction periods of fouling may have been longer than coagulation times as well as a condensate film on the glass tube and a fast rotation of the liquid within the glass tube.

Data recording was stopped when visually detectable aggregation in the sample occurred. Coagulated samples were chilled in an ice-water mixture and discarded. The maximum temperature and time of coagulation were taken from the heating curve data. HCT was calculated from the time when the samples exceeded 55 °C.

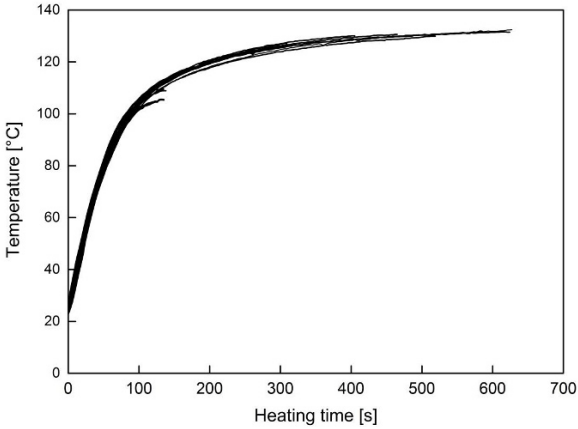

Fig. 3-2: Heating curves of individual milk samples subjected to heat stability test. Endpoints of the graphs indicate the coagulation point where measurement was stopped and data were taken for comparison of individual samples.

3.3 Results and discussion

Influence of initial pH and total solids content on heat coagulation time and temperature
Studies on the pH-dependency of HCT were largely of interest when seasonal variations and variations due to microbial count in milk occur and therefore a readjustment of pH may lead to an increase in heat stability for processing of milk and especially concentrated milk. For the sake of comparison of the maximum of heat stability of the milk used in this study with data from literature and an extension of measured skim milk total solids up to 35%, this part of the study concerning heat coagulation as a function of initial pH at 20 °C was conducted. Results for milk concentrated by evaporation up to 25% non-fat total solids were already known from literature (Muir and Sweetsur 1978). Additionally, the evaluation of the developed test method was conducted. As part of the evaluation, a test of the hypothesis that especially concentrated skim milk samples may form a coagulum already during heating of the sample to the maximum sample temperature was performed. Changes in pH due to concentration of skim milk by RO led to increasing amounts of NaOH to be added for readjustment of pH to the alkaline side with increasing total solids content as well as less amount of HCl to decrease pH for the results presented in Fig. 3-3 and Fig. 3-4.

Fig. 3-3 shows the dependency of HCT on a log scale on pH for unconcentrated milk and concentrated milk up to a total solids content of about 35% non-fat total solids, concentrated by RO. Experiments were conducted within a six months period which was possible due to low variations in composition and overall heat stability of

the skim milk used in terms of coagulation time and temperature as discussed later in this publication.

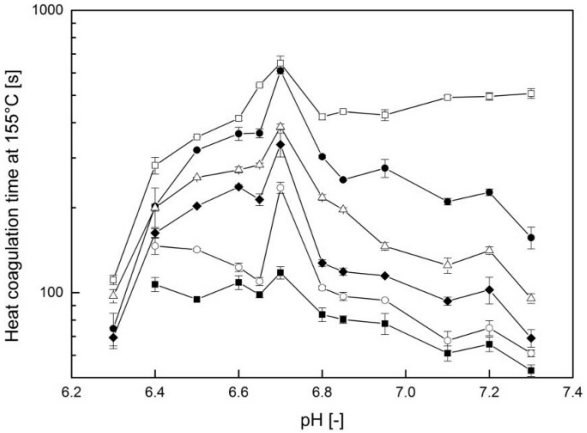

Fig. 3-3: Heat coagulation time as a function of the pH at 20 °C. Data is shown for 10% (–□–), 15% (–●–), 20% (–△–), 25% (–◆–), 30% (–○–), 35% (–■–) of total solids.

The results for HCT depending on pH already reported in literature could be qualitatively repeated concerning the HCT profile as a function of pH and extended to higher total solids content. A large headspace, which causes slight concentration of the sample during heating, was at first assumed as a major disadvantage of the procedure used. However, this evaporation is reproducible due to equilibrium of the amount of vapour and temperature of the sample. The amount of vapour was estimated to increase from 25 to 70 mg from 100 to 135 °C at 40 mL of headspace based on data of saturated steam, which is negligible small compared to approximately 11 g of sample. The amount of condensate on the glass surface may also result in a slight concentration but may also be less than 500 µL at a maximum. As a raw estimation this results in a volume reduction of 5% during heating. This fact may even shorten measured HCTs and therefore may result in lower heat coagulation temperatures and shorter coagulation times. However, relative comparison of measured values and a qualitative reproduction of published data as well as extension to higher total solids using the described test method were possible. Headspace and headspace oxygen was the same for all samples due to equal amounts of sample used for each measurement.

As shown for raw skim milk and concentrated skim milk, heat stability showed a sharp maximum at pH 6.7. A slight local minimum in HCT was detected at pH 6.8 for unconcentrated milk as reported earlier in literature. Bulk milk used in this study can therefore be classified as type A milk (Tessier and Rose 1964; Holt and Horne 1996). The minimum in the HCT-pH-profile vanished for concentrated milk at a total solids level higher than 15%. Slight differences in the location of the maxima and

minima of heat stability reported in literature might be due to differences in milk composition of raw milk or due to the use of cold concentration of skim milk by reverse osmosis at 10 °C in this study compared to evaporation at elevated temperatures. Heating of milk was shown to lead to a shift in heat stability to the acid side (Singh & Fox, 1985). Losses in urea during RO and resulting effects on heat stability were not analysed. Reconstituted skim milk prepared from low heat skim milk powder also showed maximum heat stability at pH 6.7 as reported in this study using RO concentrates (Sikand et al. 2010).

HCT decreased significantly with increasing total solids content. The effect of an increase in stability due to an increase in pH by NaOH addition to concentrated skim milk decreased with increasing total solids concentration. This is indicated by the lower maximum at pH 6.7 in Fig. 3-3 for concentrated skim milk. At an extremely low pH value of 6.3, HCT decreases sharply for all skim milk samples. At pH above 6.7, heat stability decreased for all concentrated milk samples.

Fig. 3-4 shows the corresponding heat coagulation temperature of the sample attained when visible coagulation of the sample occurred. It was shown that a decrease in HCT also leads to a decrease in heat coagulation temperature. At a low pH value of 6.3, heat coagulation temperature decreases sharply for all skim milk samples as already observed by Miller and Sommer (1940) whose results are comparable to ours and showed that this sharp decrease continues down to pH 5.2 for unconcentrated milk. They found that at pH 5.2, milk coagulates at room temperature whereas between pH 6.6 to 6.4 heat coagulation temperature is rather constant. The higher the total solids content of a sample, the lower is the temperature at the moment of visible coagulation independently of pH.

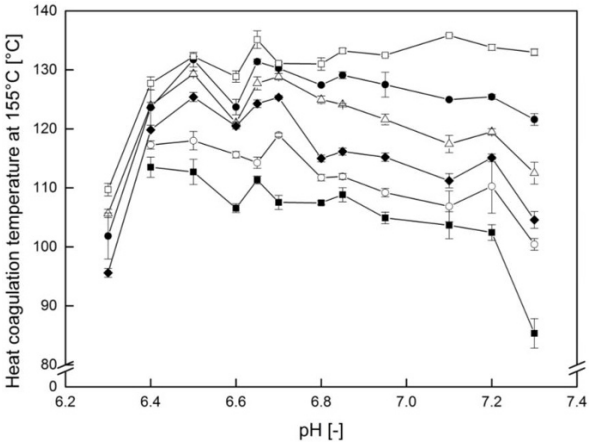

Fig. 3-4: Heat coagulation temperature as a function of the pH at 20 °C. Data is shown for 10 % (–□–), 15 % (–●–), 20 % (–△–), 25 % (–◆–), 30 % (–○–), 35 % (–■–) of total solids.

This means that samples with increasing total solids content coagulate already during the heating process of the sample prior to reaching the desired target temperature. The assumption made in most of the earlier studies that HCT at a certain oil bath temperature as a single criterion gives comparable results for different total solids levels of concentrated milk therefore seems to be questionable.
Trials on a pilot scale revealed that the maximum temperature to induce visible aggregates during heat treatment was higher than the temperature measured in the heat stability test (data not shown). Fast heating of concentrated milk by continuous flow heating equipment within seconds or few minutes does not include an extended heating-up profile to reach the maximum temperature and cooling times compared to batch heating systems. This led us to the conclusion that there might be a kinetic dependence of heating time and temperature which might lead to further insights into the molecular mechanism as well as to guidelines for practical applications, i.e. the transfer of obtained data to continuous heating systems. Reaction kinetics, which were used to describe the thermal denaturation of whey proteins, the inactivation of bacteria and bacterial spores and the formation of heat-induced reaction products in milk and other dairy products (Kessler and Horak 1981; Claeys et al. 2003; Elliott et al. 2005; Dogan et al. 2009) might also be applicable to some extent to describe heat-induced coagulation of concentrated milks.

Relationship between heat coagulation time and temperature

From the results obtained by measuring the coagulation temperature in addition to the coagulation time we assumed that these two parameters must be linked together eventually leading to coagulation of the milk samples. This is partially obvious, but in the related literature both of these criteria were always handled separately. Therefore, the oil bath temperature was varied for heat stability testing of concentrated skim milk at different total solids levels to assess whether the coagulation time changes with varying maximum sample temperatures. Heating curves up to a constant sample temperature varying oil bath temperature are shown in Fig. 3-5.

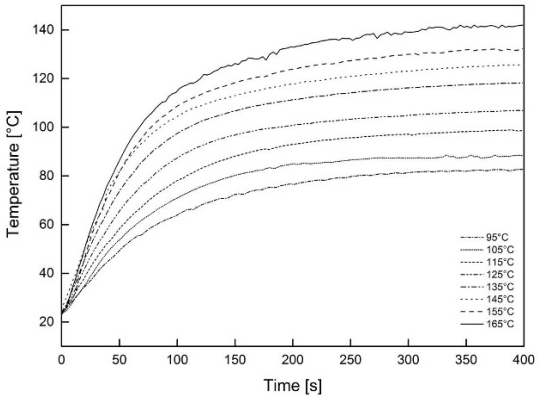

Fig. 3-5: Heating curves of samples at various oil bath temperatures.

This diagram shows that the maximum sample temperature is always approximately 18-20 K lower than the respective oil-bath temperature, independently of the oil-bath temperature chosen. Heating time to come close to the steady-state temperature was 200-250 s in all cases. The assumed reaction kinetics are strongly dependent on the heating profile, i.e. the actual sample temperature, and less on the equipment used these heating profiles are shown for the sake of characterization and documentation of the test method used in this study.

A semi-logarithmic plot of coagulation time against coagulation temperature of concentrated skim milk showed that a description of coagulation in terms of coagulation time and temperature over a broad range of total solids levels of concentrated skim milk was possible (Fig. 3-6).

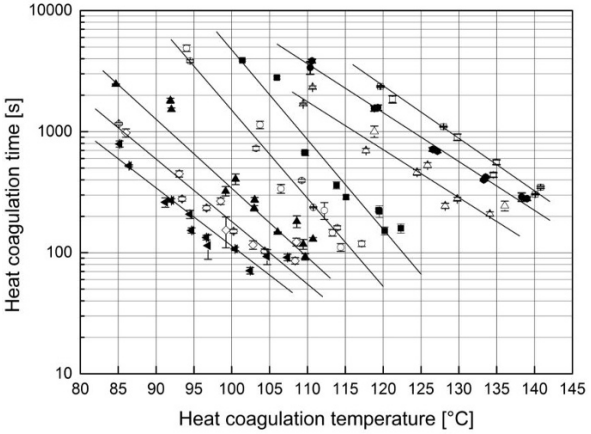

Fig. 3-6: Correlation between milk sample temperature and time of coagulation of concentrated skim milk of different total solids content. Data is shown from right to left for 10 % (□), 15 % (●), 20 % (Δ), 25 % (■), 27,5 % (○), 30 % (▲), 32,5 % (◇) and 35 % (◄) of total solids. Results of two individual trials were included in the study. All data is summarized in this graph and regression includes both replicates.

A lower sample temperature at coagulation due to a lower oil bath temperature led to an exponential increase in HCT. HCT tested at 120 °C maximum sample temperature for concentrated milk at a 20% total solids level and natural pH was in the range of data reported in literature (Singh 2004). On increasing the oil bath temperature to 165 °C, it was even possible to coagulate unconcentrated milk in a relatively short period of time. By decreasing the oil bath temperature below 120 °C, the kinetic relation of heating time and temperature at higher total solids content could be further confirmed. Regression analysis showed that a strong linear correlation exists in a semi-logarithmic plot of coagulation time against temperature. This correlation of HCT and temperature had been shown by Webb and Holm (1932) over a broad range of temperatures for unconcentrated and two-fold concentrated milk and by Davies and White (1966) for unconcentrated milk in the range of 133-137 °C. However, an

extension of the analysis to a higher total solids content of concentrated milk samples and a derivation kinetic of parameters for a transfer of the results to continuous heating systems and higher total solids levels were not in the scope of these studies.
A correlation of HCT and coagulation temperature in an autoclave heating system had also been observed by Holm et al. (1923) who investigated the effect of milk quality and sterilisation temperature on time of coagulation of skim milk and whole milk. These authors noted that the severity of heat treatment until the sterilisation temperature was reached may be higher for samples heated at higher temperatures. Higher sterilisation temperatures required longer times to obtain the final sterilisation temperature and therefore induce more chemical and physical changes during heating. A major limitation of the subjective measurement of heat stability used in the study of Holm et al. (1923) was also seen in the fact that during heating, no changes inducing coagulation can be observed. Therefore, it was impossible to quantify the effect of temperature and time prior to the coagulation of the samples. This can be seen as a limitation of all subjective oil-bath methods measuring HCT including the test method used in this study, where, however, the degree of subjectivity could be substantially reduced by vigorous shaking of the test sample and the creation of a thin film enabling an instant and better visualization of coagulation.

Therefore, slopes of the individual curves depicting the log coagulation time versus temperature relationships for each total solids level must be seen as exclusively related and applicable to the test method described with its respective heating profiles obtained. However, it is assumed that due to the long heating times in this test, true z-values of the reactions occurring until visual coagulation occurs must be lower than 20-30 K for concentrated milk as found in this study using laboratory scale batch heating. However, in continuous heating systems, heating rates are much faster. Therefore, it is finally advisable to derive kinetic parameters for description of heat-induced coagulation of casein micelles by other means avoiding long heating up times.

Nevertheless, this way of performing the heat stability test resulted in a fast and very accurate determination of heat stability as coagulation is clearly visible to the operator. For the analysis of HCT and heat coagulation temperature of concentrated milk depending on total solids content of the samples, it is advisable to use a constant oil-bath temperature. However, the dependency of heat coagulation on heating time and temperature can also be used to adjust oil bath temperature according to the total solids content of the sample to increase accuracy without changing the relative comparability of the results. Variation of results at higher total solids content than 25% may be lower when coagulation temperature of the sample is less than 115 °C, i.e. coagulation time is in the range of 300-500 s.

Determination of heat stability time and temperature of individual milk samples
All the results obtained in this study were conducted over a 15-month period. As milk is easily perishable, concentrated milk was manufactured each week from pasteurised skim milk. This would have led to additional variation in the results presented in this study. Low variation in composition (see materials and methods sec-

tion), however, led us to the conclusion that the good quality pooled bulk milk used from a local UHT milk dairy processor in south-east Germany may show low variation in heat stability. This situation might be related to less variability of cow feed today and to better hygienic condition as compared to the decades ago, when the heat coagulation test methods were developed and established as standard methods for analysis of milk quality concerning heat stability of milk and concentrated milk.

Results of HCT and temperature are shown in Fig. 3-7. Heat coagulation temperature drastically decreased as total solids content increased and so did HCT for all nine reference samples analysed over a 15-month period. An almost linear relationship exists between HCT on a log scale and coagulation temperature when results are plotted for milk and milk concentrates in one diagram.

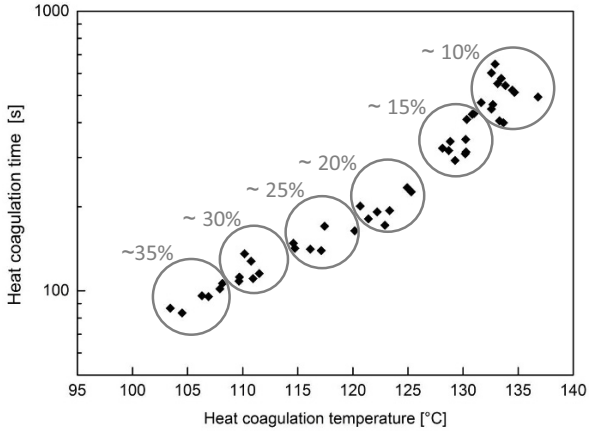

Fig. 3-7: Heat coagulation time correlated with sample temperature of individual skim milk samples over a 15 months period. Results include variation in heat stability of individual milks and subjectivity of four operators performing the heat stability test at 155 °C oil bath temperature.

Discrete skim milk total solids contents could be summarized in heat stability categories. This fact may allow the analysis of skim milk for heat stability and thereof estimate the resulting heat stability of concentrated skim milk of various total solids content before large-scale concentration and further processing. The less concentrated samples had a higher heat stability indicated by coagulation time and temperature. Bulk milk used in this study showed a coagulation temperature in the range of 131–136 °C at a corresponding time of coagulation from 450 to 600 s. Concentrated milk at a total solids level of 30% total solids showed a coagulation temperature of about 110 °C and a coagulation time of 100-150 s calculated from the sample reaching 55 °C. This means that it would even not be necessary to concentrate milk for heat stability testing. Skim milk of good quality can be measured as is and coagulation time and temperature obtained for milk can be used to predict the heat stability of individual milk at a certain total solids level. Differences in overall heat stability of

the individual milk samples were small and an increase in total solids content was seen to be the dominating effect. An increase in soluble calcium and magnesium, a decrease in pH, an increase in ionic strength proportional to volume reduction and an increase in total protein are supposed to be the major variables limiting heat stability of concentrated milks with increasing total solids content (Holm et al. 1932; Morrissey and O'Mahony 1976; Holt et al. 1978a; Dalgleish et al. 1987; Augustin and Clarke 1990; Pouliot and Boulet 1991; Le Ray et al. 1998; Faka et al. 2009; Anema 2009; On-Nom et al. 2012).

As this test method is based on subjective endpoint determination as titration methods are, a slight variation due to visual determination of coagulation is likely to be included in the results. But it must also be noted that, at higher oil-bath temperatures, changes in the samples within a few seconds are massive when coagulation occurs. This was also shown by White and Davies (1966) measuring sedimentable nitrogen during coagulation of concentrated milk. Hence, this subjective aspect is not supposed to be of major significance compared to sample composition. Lehmann and Buckin (2005) also showed that high resolution ultrasonic spectroscopy as an objective test method gave comparable results to a subjective oil-bath method which demonstrates that subjective methods might be the method of choice when simple and cheap equipment is preferred to be used.

3.4 Conclusion

Novel insights into the relation of heating time and temperature of concentrated milk for heat-induced aggregation are presented. Determination of heat stability with the newly developed test assembly was fast and reproducible, the coagulation was easy to recognize and results were reliable, especially at high total solids level. A strong dependency of the pH of milk and concentrated milk on both, total solids content and temperature of the concentrate, was observed. Results from earlier publications on pH-dependency of heat stability could be reproduced and extended to a higher total solids level. Measuring HCT and coagulation temperature made it possible to establish first insights into a kinetic correlation between these two physical parameters, even at high total solids contents of concentrated skim milk which needs to be further addressed. A transfer of these insights to continuous flow heating equipment will potentially lead to optimized heating processes to heat treat milk concentrates with much shorter times of exposure to higher temperatures and, thus, to a gain in importance of liquid concentrates for powder replacement by liquid milk concentrates in the dairy industry. Determination of HCT and temperature of concentrated skim milk over a 15 months period led to the conclusion that the variation in heat stability of the good quality pooled bulk milk used was low and that heat stability can be estimated from the heat stability results obtained for unconcentrated milk as an increase in total solids was seen to be the predominant effect on heat stability of milk.

Summary and contribution of the doctoral candidate

The industrial processing of milk and concentrated products thereof almost inevitably includes one or more heat treatments in combination with other processing steps. Heat treatments are used either for targeted restructuring of milk or inactivation of enzymes and microorganisms to extend the shelf. Temperature-time combinations used for heating will be chosen according to the desired shelf life and storage temperature. This is usually done by using the inactivation kinetics of the target organism and enzymes, setting a threshold for residual amounts, and the design of the temperature-time profile necessary to meet these requirements. Unconcentrated milk was found to be highly heat stable and will withstand severe heat treatments for sufficient enzyme inactivation and sterilisation without heat-induced destabilisation of casein micelles leading to coagulation, gel, aggregates, and sediment formation. However, concentrated milk shows reduced heat stability and was often found not to withstand UHT or in-container sterilisation regimes required for commercial sterility depending on pre-processing conditions and equipment used. Pre-processing conditions and compositional aspects had been investigated in numerous studies. However, the major lack in knowledge was if there is a dependency of heat-induced coagulation of milk and concentrated milk on the severity of the heat-treatment in terms of temperature and time combinations used and the total solids content of the concentrate. This was the hypothesis of this study together with the assumption that heat stability of concentrated milk is also dependent on the total solids content including changes in pH during concentration.

For this purpose, a laboratory scale heat stability test was developed that allowed for the investigation of the relationship between the total solids content of the concentrated skim milk and the heat stability of the resulting concentrate in terms of temperature-time relationships up to 35% total solids. The decrease in pH during concentration of milk by reverse osmosis (RO) was a second aspect that needed to be addressed as heat stability had been found to be strongly dependent on pH and there were no data on the pH-dependency of heat stability of RO concentrated skim milk, especially at high total solids content.

The major outcome of this study was that the heat stability of concentrated skim milk can be kinetically described a function of temperature, time, and total solids content using a modified subjective heat stability test. Lines of equal effect for the onset of coagulation could be derived on a semi-logarithmic plot that clearly demonstrate the close relationship between coagulation temperature, coagulation time and the total solids content of the concentrate. This was possible due to the variation of the oil-bath temperature which resulted in the heat coagulation temperature at a certain total solids content. This variable oil bath temperature was the key to understand this relationship which shows the conflict between heat stability and microbial inactivation, and to estimate the heat stability of concentrated milk.

The substantial contributions of the doctoral candidate were given by the literature review based on which the concept and design of the heat stability test was de-

veloped. Creation of templates, data collection, analysis, evaluation, and representation was carried out by the doctoral candidate. The manuscript was mainly written and revised by the doctoral candidate.

4 Heat stability of concentrated skim milk on pilot scale[7]

Abstract

Direct steam injection heat treatment on pilot scale as an alternative to lab scale indirect heat treatment was applied to investigate heat stability of concentrated skim milk across a broad range of temperatures from 117 °C to 153 °C and from 0.5 to 13 s holding time, assessing options for heat treat treatment of concentrated skim milk without a significant amount of protein sediment formation. The relationship between total solids content of concentrated skim milk and temperature-time combinations of heat treatment could be established using minimal heat-induced coagulation as a criterion. Coagulation of destabilized casein micelles was shown to proceed nonlinear over heating temperature. Transition of critical temperature-time combinations resulted in a marked increase in sediment formation indicating that preceding reactions, noticeable as the formation of dissociated material, need to take place to some extent to induce coagulum formation. UHT preheat treatment of skim milk prior to concentration was shown to increase heat stability in terms of possible temperature-time combinations without coagulation.

7 Original publication: Dumpler J, Kulozik U (2016) Heat-induced coagulation of concentrated skim milk heated by direct steam injection. Int Dairy J 59:62–71. doi:10.1016/j.idairyj.2016. 03.009. Adapted original manuscript. Adaptions of the manuscript refer to title, enumeration type, citation spelling, notation of units, and format. Permission for the reuse of the article is granted by Elsevier Limited.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_4

4.1 Introduction

The effect of UHT heating conditions has been studied intensively for unconcentrated milk concerning chemical and physical changes occurring during heat treatment. Physical and chemical changes of whey proteins and casein micelles, degradation of valuable components in the serum phase as well as the inactivation bacterial and endogenous enzymes of bacterial spores were investigated over the last 70 years and comprehensively reviewed (Burton 1984; Dannenberg and Kessler 1988; Bastian and Brown 1996; Claeys et al. 2003; van Asselt and Zwietering 2006; Chavan et al. 2011; Loveday 2016).

However, regarding the heat stability of concentrated milk under commercial in-line sterilisation conditions, little data exist on possible temperature-time combinations and total solids content of concentrated skim milk (CSM) without causing extensive protein aggregation and sediment formation. Typical temperature-time combinations are rather published as empirical guidelines for evaporated milk manufacture (Muir 1984; Smith and Malmgren 1999). In-line sterilisation of concentrated milk can therefore still be considered as challenging compared to in-container sterilisation. The major problem is that heat stability of concentrated milk can be lower than the processing conditions necessary for inactivation of bacterial spores resulting in coagulation and sediment formation in evaporated milk products. Heat instability of CSM will result in particle formation during heat treatment, gelation, and excessive sediment formation during storage (Muir 1984; Smith and Malmgren 1999; Hinrichs 2000; Kasinos et al. 2014). Preheating, i.e. a preheat treatment of milk prior to concentration and sterilisation of the concentrated milk is widely used for stabilisation of the concentrate against heat-induced physical changes during UHT sterilisation (Muir 1984; Smith and Malmgren 1999; Hinrichs 2000). Newstead et al. (1979) showed that differences in composition of milk as well as the sequence of processing steps affect the effectiveness of preheating. However, no quantitative correlation could be established between physical changes in milk and an increase in heat stability. In addition, the effectiveness of preheating of unconcentrated milk under UHT sterilisation conditions in terms of an increase in heating temperature and duration of concentrated milk without excessive sediment formation has not been assessed so far.

At present, minimally processed microbial stable and safe milk concentrates become increasingly important as intermediate products prior to spray drying or even as an energy efficient alternative to powder manufacture. More recently, it could be shown that heat stability of concentrated milk decreases in terms of heating temperature-time conditions at elevated total solids content (Dumpler and Kulozik 2015). Relatively little is known about the extent and mechanisms of physical changes in concentrates, especially milk concentrated beyond evaporated milk that had undergone temperature-time conditions of UHT heat treatment. Moreover, data regarding the heat effect of direct heat treatment at ultra-high temperature and very short holding times (<1 s) of concentrated milk has not been published to our knowledge.

There have been attempts to describe the destabilising reactions occurring in milk and concentrated milk investigated by numerous heat stability tests, mostly according to the subjective test method established by Davies and White (1966) and the objective test developed by Whitney et al. (1952) and further improved by White and Davies (1966). Different mechanistic explanations for heat-induced aggregation of casein micelles based on analytical data as well as electron microscopic studies were proposed both for unconcentrated milk and concentrated milk. White and Davies (1966) determined the amount of sedimentable protein for different milk heating times at a constant temperature and concluded that heat-induced coagulation is a two-step process which involves an induction phase during which particles may increase to a certain extent and a slow or fast coagulation step depending on the pH and composition of milk prior to heating. However, they stated for their own study that centrifugation condition for removal of sedimentable protein were chosen rather arbitrarily as, at that time, they could not measure particle sizes of casein micelles and aggregates. It was therefore not possible to prove a complete sedimentation of aggregated particles. They concluded that a variation in centrifugation conditions as well heat treatment and milk protein content will result in different kinetic and thermodynamic parameters affecting and characterizing this aggregation reaction. Insights into the mechanism of heat-induced coagulation were gained somewhat later by White and Sweetsur (1977) for unconcentrated and modified milk under in-container sterilisation conditions. A decrease in pH due to heat-induced degradation of lactose, calcium phosphate precipitation, Maillard browning as well as cleavage of phosphoserine residues was considered as reasons for heat-induced destabilisation of casein micelles. However, these effects were found to be non-correlating to the onset of coagulation of caseins in milk (Sweetsur and White 1975; van Boekel et al. 1989a).

Therefore, the aim of this study was to use direct steam injection (DSI) heat treatment of concentrated skim milk (CSM) to extend the relationship of CSM total solids, temperature and heating time on the onset of heat-induced coagulation found by Dumpler and Kulozik (2015) to industrial scale. This was necessary due to the fact that temperature-time relationships of lab scale experiments were not directly applicable on pilot and industrial scale as heating profiles are different.

Direct steam injection also offers the opportunity to investigate mechanistic aspects of the resulting coagulation process due to a fast heating and cooling of CSM without intensive chemical effects resulting in a decrease in pH due to lactose degradation, cleavage of phosphoserine residues and Maillard-browning which were considered at least as contributing to heat-induced coagulation of milk protein (Pyne and McHenry 1955; Rose 1961b; Sweetsur and White 1975; van Boekel et al. 1989a; Nieuwenhuijse et al. 1991). Eliminating more and more interfering chemical reactions will lead to a deeper insight into the coagulation process itself. Chemical reactions may be regarded rather as interfering.

4.2 Materials and methods

Preheating and concentration of skim milk by reverse osmosis (RO)

Pasteurised (74 °C for 28 s) skim milk was obtained from a local dairy factory (Molkerei Weihenstephan, Freising, Germany). Pasteurised skim milk was concentrated by reverse osmosis (RO) on a pilot scale at 9-10 °C at a transmembrane pressure of 45-50 bars up to the desired total solids content without further storage. The accurate total solids content was adjusted by adding a small amount of RO permeate if necessary. For comparison, some experiments were conducted with UHT preheating of skim milk prior to concentration on an indirect tubular pilot plant heat exchanger (GEA TDS GmbH, Ahaus, Germany). Milk was preheated at 90 °C for 80 s, then heated at 142 °C for 5 s and cooled to 10 °C for concentration by reverse osmosis. The pH of concentrated skim milk before heat treatment is indicated in Tab. 4–1. The pH of concentrated milk was not significantly different after direct steam injection heat treatment. Variations in pH partly resulted from slight changes in water content of CSM before and after heat treatment. Preheat treatment of unconcentrated milk prior to concentration resulted in an average decrease in pH of concentrated milk at 27% total solids of 0.02 pH units. The reverse osmosis membranes used for concentration of skim milk had a salt retention >99.2% (DOW FILMTEC™ SW30-2540, FilmTec Corporation, Edina, MN, USA). Reduction of urea levels in CSM was not monitored.

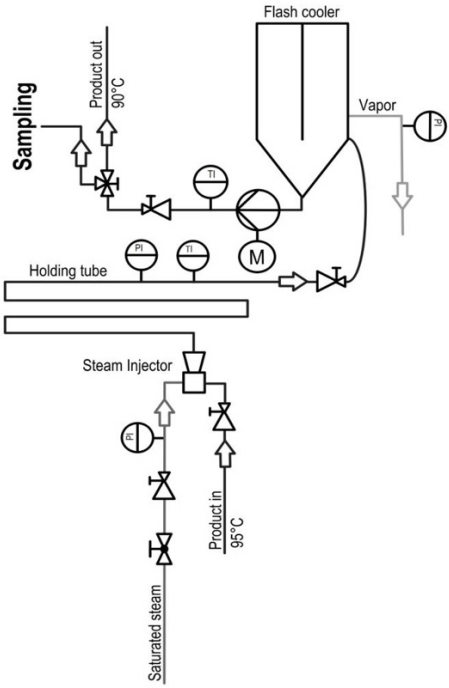

Fig. 4-1: Simplified P&I diagram of the pilot plant unit for direct steam injection heat treatment of concentrated skim milk preheated to 95 °C by indirect heat exchange prior to steam injection. Sample aliquots were taken immediately after flash evaporation of the concentrate.

Heat treatment of concentrated skim milk using pilot scale direct steam injection

Batches of 40 L of CSM were used for each trial using pilot scale steam injection heat treatment. A simplified P&I-diagram of the direct steam injection unit is shown in Fig. 4-1.

Tab. 4–1:The pH values of skim milk and concentrated skim milk (CSM) before heat treatment.

Initial total solids TS_0 (%)	$pH_{20°C}$ before heating[a]
31.5	6.42
28.5	6.45
27	6.47
26	6.48
24	6.51
20	6.56
18.5	6.59
15	6.65
12	6.71
9.6	6.75

[a]values are the mean of two different batches.

Preheating of the concentrate to 95 °C before steam injection was performed by indirect heat exchange. A temperature range from 149 to 117 °C was 'scanned' in steps of 4 °C in the case of non-preheated CSM and 153 to 121 °C in the case of preheated CSM using direct steam injection. The in-house designed steam injection system used showed a minimum of cavitation and therefore little shear stress on the product, no noise and a constant condensation process of saturated steam in CSM. The temperature-time profile of the pilot plant is shown in Fig. 4-2. Indirect preheating temperature was 95 °C. Direct steam injection causes the temperature to rise to the target temperature in less than 1 s. The holding time was adjusted by the length of a stainless steel tube including a back pressure valve as well as a pressure and temperature sensor at the end of the tube. Flash cooling temperature was 90 °C for removal steam condensate by an equal amount of vapour. Samples were drawn immediately after flash cooling of the concentrates in a vacuum chamber at about 750-800 mbar. Falcon tubes were tightly closed and immediately cooled further in an ice-water mixture. Sampling was possible every 3-4 min after manual readjustment of the saturated steam pressure, equilibration of heating temperatures and removal of the mixing phase of product heated at different temperatures from the tubes. This experimental technique resembles to some extent the approach of Miller and Sommer (1940). In a first series of trials, a variation of total solids of CSM was performed in the range from 12% to 31.5% total solids. Samples of 12% total solids were not analysed as no coagulation could be detected throughout the temperature range from 117 to 149 °C at a constant holding time of 9.5 s. In a second series of experiments, we investigated the effect of the holding time in the range from 0.5 to 13 s on heat coagulation of CSM with a constant total solids level of 27% over the entire temperature range mentioned above. In a third series of experiments, we investigated the effects of the indirect UHT preheating of skim milk on the heat-induced aggregation of proteins in CSM prepared thereof and heated by direct steam injection. All trials were repeated twice and at an individual total solids-temperature-holding time set two individual samples of 50 mL were drawn in Falcon tubes and used for analysis. Standard deviations of the results were calculated from these four samples.

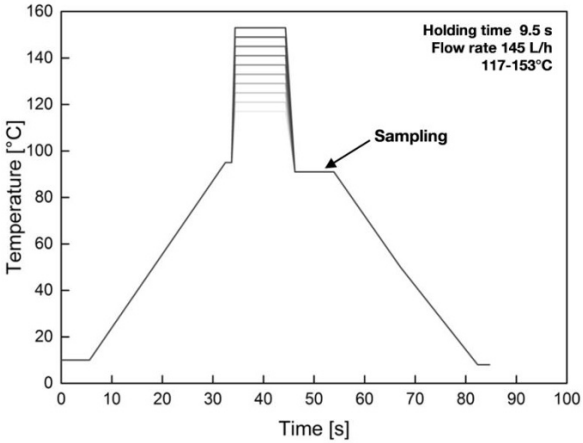

Fig. 4-2: Temperature-time profiles of the direct steam injection ultra-high temperature (UHT) pilot plant. Final product temperature was varied in steps of 4°C by adjustment of the steam pressure.

Scanning electron microscopy

Heat treated and unheated CSM of 27% total solids was diluted 1:3 with distilled water. Approximately 20 µL of diluted sample were spread over a glass slide (25 x 75 mm) and dried under a gentle flow of filtered air within 2 min. Glass slides were then cut into smaller pieces and sputter coated for 90 s with gold in an argon plasma (10-11 nm) with a BAL-TEC SCD 005 sputter coater (Bal-Tec AG, Liechtenstein). Scanning electron micrographs were obtained by a JEOL JSM-5900 LV Scanning electron microscope (JEOL GmbH, Eching, Germany) at 15 kV. Deliberately, no further sample preparation like fixation or exchange of solvents was used for sample preparation.

Separation of protein aggregates by centrifugation

Preliminary trials had shown that centrifugation of 50 mL Falcon tubes with a sample level of 8 cm (approximately 40 g) of undiluted CSM at 4,000*x*g for 10 min using a laboratory centrifuge (Multifuge 1S-R, Heraeus Holding GmbH, Hanau, Germany) results in a complete removal of protein aggregates from the non-aggregated casein micelles formed during heat treatment by direct steam injection. These centrifugation conditions were applied to all samples analysed independently of total solids, heating temperature and holding time as protein aggregates were seen to be distinctly larger than non-aggregated proteins. Changes in viscosity due to different initial total solids content as well as due to whey protein denaturation and voluminosity changes of casein micelles did not affect the sedimentability of large particles significantly. Viscosity of heat treated samples was maximal at the onset of coagulation and decreased when heat-induced aggregation occurred.

Particle size analysis

Particle size of the heat treated samples was measured directly after heat treatment as well as after centrifugation by laser light diffraction using a Malvern Mastersizer 2000 equipped with a Malvern Hydro 2000S sample dispersion unit (Malvern Instruments GmbH, Herrenberg, Germany). Refractive index of the dispersant (softened water) was set at 1.33 and the refractive index of protein was set at 1.41. Particle absorption index was set to 0.001. The sample was added and dispersed at a constant stirrer speed until an obscuration between 10 and 20% was obtained according to the guidelines of the manufacturer. Each sample was measured in duplicate at 20 °C within 3 min. A dissociation of protein particles due to dilution of the sample in softened water noticeable as a change in particle size distribution was not observed when the samples were diluted 30 minutes before measurement was performed.

It was suspected that during aggregation of casein micelles to form large particles, small particles of casein micelle fragments might also form that would not be detected by the Mastersizer as the detection limit of this instrument was about 50 nm. Therefore, supernatants were diluted 1:50 in simulated milk ultrafiltrate (SMUF) according to Jenness and Koops (1962) and measured by photon correlation spectroscopy (PCS) in a Malvern Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). Samples were measured in 173° backscatter mode at 20 °C eight times for 1 min after an equilibration time of 120 s. Results of the particle size measurements reported in this study are means of eight measurements of two individual samples and two individual trials. In this case, SMUF was chosen as a dispersant due to a much longer measurement period and possible changes in particle size following the method of O'Connell and Fox (2000). Data were transformed into a logarithmic density distribution q_3 [ln(x)] to get an impression of the relative volume quantities of large and small particles of the bimodal distributions observed.

Determination of protein content and of the degree of denaturation of whey proteins

Crude protein content was determined according to the method of Dumas using a vario MAX cube (Elementar Analysensysteme GmbH, Hanau, Germany). Aspartic acid was used for calibration of the system. Wheat flour was used as a reference sample. Approximately 500 mg of centrifugal supernatant were weighed into a crucible and burned at 900 °C. Helium was used as a carrier gas. Resulting exhaust gases were reduced and purified and analysed for elementary nitrogen with a heat conductivity detector. A nitrogen conversion factor of 6.38 for milk proteins was used. Sedimentable protein S_p was calculated by

$$S_p\ (\%) = \left(1 - \frac{C_{T,t}}{\overline{C_0}}\right) \cdot 100 \qquad (4.1)$$

where $C_{T,t}$ is the concentration of protein in the supernatant heated at the temperature T and time t and $\overline{C_0}$ is the mean of the two unheated samples analysed for each batch of CSM.

The degree of denaturation in CSM of different total solids was determined by precipitation the pH 4.6 insoluble material with 1 N HCl after 1:7 dilution of CSM and analysis of the supernatant by RP-HPLC as described by Toro-Sierra et al. (2011). The degree of denaturation was calculated similar to eq. (1).

Data regression and statistical analysis

Data was plotted and data regression of sedimentable protein was performed using OriginPro 2015G (OriginLab Corporation, Northampton, MA, USA). Error bars represent standard deviation of four samples from two replicates of the pilot trials. The 2% level of sedimentable protein of total protein was determined by regression analysis using a Gompertz equation for regression of the percentage of sedimentable protein

$$S_p\,(T) = S_{p,max} \cdot \exp(-\exp(-k \cdot (T - T_c)) \tag{4.2}$$

where $S_{p,\,max}$ is the maximum percentage of sedimentable protein at a constant holding time, k is a constant and T is the actual heating temperature, and T_c is the temperature of the inflection point $\left(T_c / \frac{S_{p,\,max}}{e}\right)$. *z*-Values were determined from the regression analysis of sedimentable protein over holding time at a sedimentable protein level of 2% using the boundary condition that $\lim_{t \to 0} f(t) = \infty$ and therefore we assumed an exponential equation for regression analysis.

4.3 Results and discussion

Heat-induced changes in casein micelles as observed by scanning electron microscopy

As a first example of electron micrographs of heat treated CSM of 27% total solids at 145 °C and 6 s holding time, illustrated in Fig. 4-3, show that coagulation results in large proteinaceous particles (A). These large spherical particles of 10-20 µm further aggregate to even lager non-spherical particles as could be seen when the magnification was further increased (B). At the surface of these spherical particles, distinct casein micelles merged into the particle surface can be detected (C). It is obvious from these electron micrographs that during heat-induced coagulation, casein micelles merge into large aggregates causing the aggregates to grow. An intermediate state of only few aggregated casein micelles was scarce and only observed at holding times of less than 2 s. From this single situation presented in Fig. 4-3 of only one single CSM obviously destabilized by the heat treatment applied, it was not clear how these large particles may form from virtually intact micelles. Therefore, we further investigated CSM of 27% total solids over the entire range of temperatures applied at a constant holding time of 6 s by SEM. it was assumed that there may be changes induced by heat that precede coagulation, i.e. the formation of large particles from casein micelles.

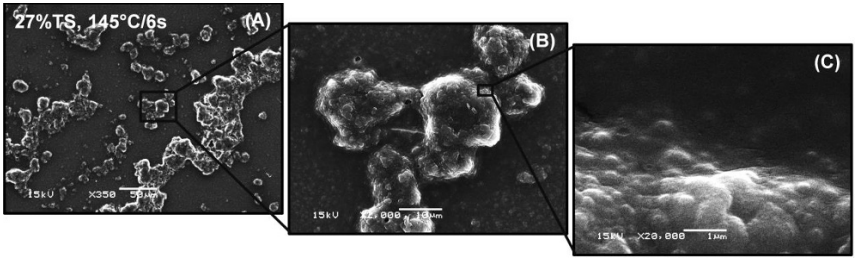

Fig. 4-3: Scanning electron micrographs of protein particles formed during direct steam injection heat treatment of concentrated skim milk at 145 °C. Magnification increases from left to right. (A) 350x, (B) 2,000x, (C) 20,000x.

Fig. 4-4 shows the supernatant after removal of large aggregates that form at higher temperatures than 129 °C for 27% total solids content and holding time. It shows that an increase in temperature by indirect heating without steam injection first causes the casein micelles to become larger (B) compared to the unheated sample where casein micelles are rather small distinct particles (A). Additional heating by direct steam injection, shown in pictures (C) to (E), causes the casein micelles to grow further without noticeable formation of large aggregates. Casein micelle average diameter ($d_{50,3}$) increases by a factor of ~ 2 before sedimentable particles emerge.

Above 133 °C, coagulation occurs that results in the formation of the aforementioned protein agglomerates containing large particles that were removed by centrifugation at 4,000*x*g/10 min. We found the residual non-aggregated casein micelles to increase further in size whereby the contours of the non-aggregated micelles became increasingly blurred (Fig. 4-4 (F) - (I)). Increasing heating temperature not only caused whey proteins to denature but also casein micelles to lose their native structure due to irreversible dissociation reactions induced by heat, low pH and therefore an increased calcium activity as well as high ionic strength. A reduction in packing density of the casein micelles, stronger hydration of the internal structure will result in this detectable increase in casein micelle hydrodynamic radius. This increase in voluminosity cannot only be explained by adsorption of whey proteins to casein micelles as the degree of whey protein denaturation at 121 °C heated for 6 s was about 80% and was also found in concentrated milk made of UHT preheated milk. This increase in micelle size after heat treatment of concentrated milks has often been observed by electron microscopic studies and was ascribed to an adsorption of whey proteins to the micellar surface (Nieuwenhuijse et al. 1991; Tran Le et al. 2008; Donato and Guyomarc'h 2009), a fusion of small casein micelles with larger ones (Aoki et al. 1977) or no explanation was given (Hostettler et al. 1968; Dalgleish et al. 1987; Nieuwenhuijse et al. 1991; O'Connell and Fox 2000). The degree of denaturation of major whey proteins α-lactalbumin, β-lactoglobulin A and B before the onset of coagulation (5) of CSM of different in total solids content was in the range of 81 to 87%. No correlation between heat-induced coagulation and the degree of denaturation at the onset of coagulation could be established as the degree of denaturation proceed-

ed rather linearly with increasing temperature compared to the sharp increase in sedimentable protein at the onset of coagulation.

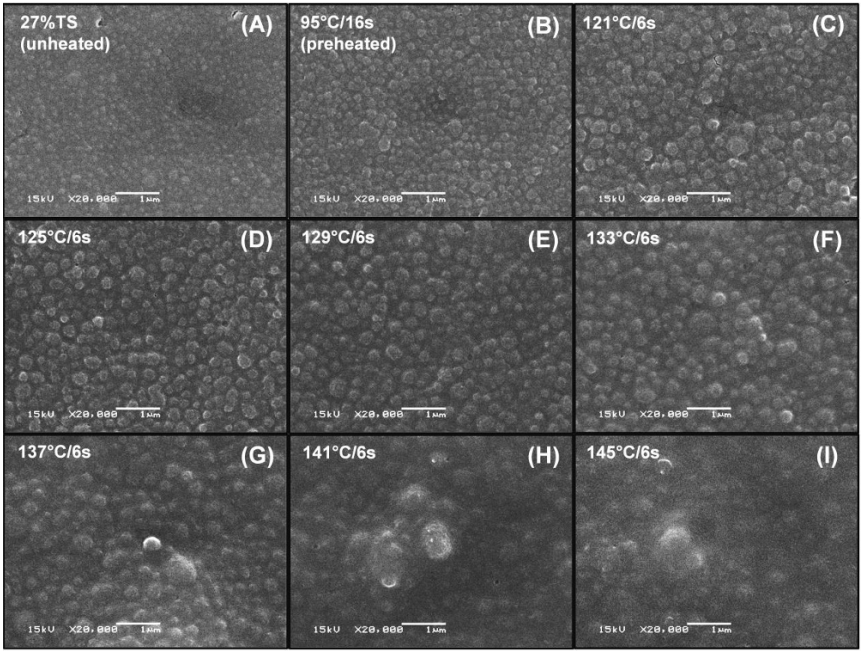

Fig. 4-4: Scanning electron micrographs of supernatants of concentrated skim milk. Unheated (A), indirect heat treated without steam injection (B) and heated by direct steam injection at different temperatures from 121 °C (C) to 145 °C (I) at a constant holding time of 6 s. Magnification of all pictures was kept constant at 20,000x.

Therefore, we conclude that a loss in structural integrity of the casein micelles, observable as an increase in voluminosity, may be a preceding step in heat-induced coagulation of especially concentrated milk. Concentrated milk systems were shown to be prone to dissociation, mainly κ-casein dissociates from the micelles (Aoki et al. 1974; Singh and Creamer 1992; Singh et al. 1995). The blurring of the electron micrographs is assumed to result from non-sedimentable small protein particles that accumulate in the serum phase as also observed by Aoki and Kako (1983) as well as Donato and Dalgleish (2006). The composition of this protein fraction needs to be further addressed.

Particle size distribution

A more quantitative description of the obvious changes in particle size of casein micelles and the emerging large particles during coagulation of concentrated milk was possible by particle size measurements using laser light diffraction. Fig. 4-5 (A)

shows a selection of measured logarithmic density distributions and shows that, in fact, there are only two groups of distinct particles.

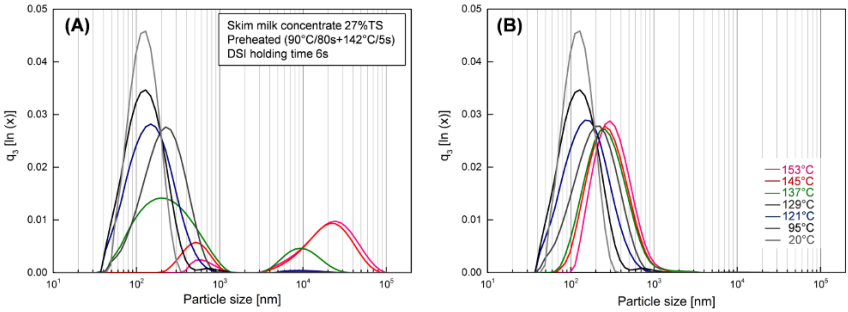

Fig. 4-5: Logarithmic density distribution $q_3[\ln(x)]$ measured by laser light diffraction of concentrated skim milk (27%TS, preheated milk) heat treated by direct steam injection at different temperatures. Large protein aggregates included in the distribution (left) could be completely removed by centrifugation at 4,000*x*g for 10 min. Particle size distributions of the resulting supernatant is shown on the right.

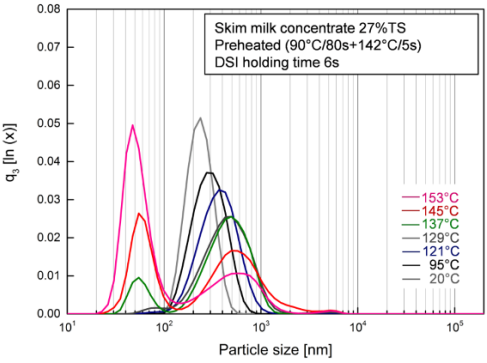

Fig. 4-6: Logarithmic density distribution $q_3[\ln(x)]$ of the supernatant from preheated concentrated skim milk (27%TS) heat treated by direct steam injection at different temperatures measured by photon correlation spectroscopy (PCS).

Particles in the range of 40-1,000 nm, representing non-aggregated casein micelles, were observed and particles ranging from 3 to 100 µm of protein aggregates. Heat treatment of preheated CSM of 27% total solids at different temperatures for 6 s resulted in these two distinct fractions of particles both increasing in size with increasing heating temperature. These results are representative for all other temperature-time combinations and total solids investigated. Results for milk that had not been preheated were similar. Fig. 4-5 (B) shows that a complete sedimentation of aggregated material from CSM was possible by the centrifugation conditions chosen due to the large aggregates formed by direct steam-injection heat treatment. No large particles were detectable in the supernatant of heat treated CSM shown in Fig. 4-5 (B).

Before the onset of coagulation, an increase in the average diameter of non-aggregated micelles could be observed as well as a broadening of the particle size

distribution. Hougaard et al. (2009) also observed a broadening in particle size distribution and increase in average diameter of casein micelles in unconcentrated skim milk heated above 100 °C for 0.2 s by instant infusion pasteurization. However, in unconcentrated milk, heat-induced aggregation of casein micelles using these heating conditions was not observed.

From the electron micrographs we also suspected smaller particles to be formed that could not be measured by laser light diffraction. So we also measured the obtained supernatants by PCS. Logarithmic density distributions for the same supernatants analysed by laser light diffraction in Fig. 4-5 (B) are shown in Fig. 4-6 analysed by PCS. A bimodal distribution was observed in the supernatant showing an increasing relative proportion of small particles next to a decrease in size of non-aggregated casein micelles. These small particles became detectable just before the onset of coagulation indicating desorption of proteinaceous material from the micelles at 121°C/6 s. These small particles were in the range between 20 to 100 nm in size for all total solids contents independent of preheating as also observed in unconcentrated milk by Donato and Guyomarc'h (2009). The results of the particle size distribution show that during heat-induced coagulation of CSM, there is a heat-induced disruption of native casein micelles increasing in size into large particles and small fragments of non-coagulable protein. The composition of these small distinct fragments of casein micelles needs to be further addressed in more detail in further work. This increase in voluminosity, weakening of the internal structure and desorption of proteinaceous material from the surface of casein micelles is obviously the preceding or concurrent step in heat-induced coagulation and of kinetic nature. A kinetic description of this voluminosity increase as well as κ-casein dissociation could not be derived in this study due to the limitations in holding time using DSI systems. Heat-induced aggregation of these damaged micelles due to an increase in attractive forces of calcium-sensitive micelles is then a second step (Singh et al. 1995). This aggregation reaction was already shown by some researchers to be of a kinetic nature using indirect lab scale batch heating systems (White and Sweetsur 1977; Darling 1980; van Boekel et al. 1989b; Nieuwenhuijse et al. 1991; Nieuwenhuijse et al. 1992).

Determination of heat stability limits of concentrated skim milk at different dry matter levels

CSMs of high total solids are still much more heat stable when compared to protein systems containing only globular proteins, where denaturation and aggregation lead to extensive sedimentation already far below 100 °C. The inactivation of heat labile vegetative microorganisms up to high total solids levels is likely to be possible due to the high heat stability of casein micelles even in concentrated systems. The kinetics of the aggregation reaction of casein micelles in a temperature range relevant for spore inactivation should now be proven by the determination of the amount of aggregated protein over total solids, heating temperature and holding time. Thereby, it would be possible to derive working areas for temperature-time combinations, where inactivation of microorganisms and bacterial spores takes place while damage in the colloidal structure of casein particles can be limited. Moreover, the impact of a decrease in pH due to lactose degradation, cleavage of phosphoserine residues and

Maillard reaction can be avoided. The kinetics of these reactions may rather interfere stabilising or destabilising with the mechanisms of heat-induced coagulation of casein micelles.

Fig. 4-7 (A) shows the amount of sedimentable aggregated protein depending on heating temperature and total solids content and Fig. 4-7 (B) shows it depending on holding time at a total solids content of 27%. 2% of aggregated protein of total protein was indicated in the diagrams as a threshold level. It can be seen from the graphs that the onset of coagulation, indicated by the sharp increase in sedimentable protein, is dependent on total solids (A) as well as on holding time (B). This suggests that these three factors together determine the heat stability of CSM. Coagulation itself was seen to proceed non-linearly with temperature in all cases. This situation was best fit by a Gompertz equation (eq. 2). There was an exponential increase in coagulated protein at a certain temperature. This means that exceeding the heating temperature by only a few degrees centigrade caused a transition from a stable colloidal system into a largely aggregated 'sandy' concentrate texture that was yellowish in appearance after heat treatment. The exponential increase of aggregated protein is limited by the maximum amount of sedimentable protein at a certain holing time and therefore tending towards a maximum. A comparison of Fig. 4-7 (B) and (C), representing 27% and 23% totals solids, respectively, shows that the onset of coagulation as well as the maximum amount of sedimentable protein at a constant holding time is dependent on total solids content.

By comparing Fig. 4-7 (B) and (D), representing CSM of 27% total solids and 27% total solids of preheated milk, we concluded that UHT preheating of milk prior to concentration resulted in a shift in the onset of heat-induced coagulation to higher temperatures at a constant holding time. As a result, there was a marked reduction of the amount of sediment at a certain temperature-time combination by preheat treatment of milk prior to concentration. This means, preheating reduces the reaction rate of the reaction preceding the onset of coagulation. In addition, a reduction of holding time below 1 s leads to noticeable prevention of aggregation of casein micelles as holding times may be too short for the aggregation of casein micelles in concentrated milk. Therefore, a reduction of holding times to below 1 s will significantly limit aggregated protein formation, but inactivation reactions were shown to proceed and therefore sterilisation of CSM or at least inactivation of pathogenic spore formers is likely to be possible (van Asselt et al. 2008; Rauh et al. 2014). The higher percentage of small protein aggregates, at 27% total solids and 0.5 s about 5% at 149 °C in this study, may be tolerable if concentrates are homogenized and immediately spray dried. However, coagulation to this extent can be regarded as undesired in the case of shelf stable liquid CSM manufacture.

Parameters of the Gompertz fits in Fig. 4-7 are shown in Tab. 4–2 for CSM of different total solids and in Tab. 4–2 for different holding times. Interpretation of the data fitted by the Gompertz equation led to temperatures T_{crit} in combination with holding time that are feasible without visible coagulation of CSM using 2% of aggregated protein of total protein as a maximum. These Gompertz-parameters can be

used to calculate critical temperatures, holding times or total solids of CSM that will not cause coagulation to a noticeable extent.

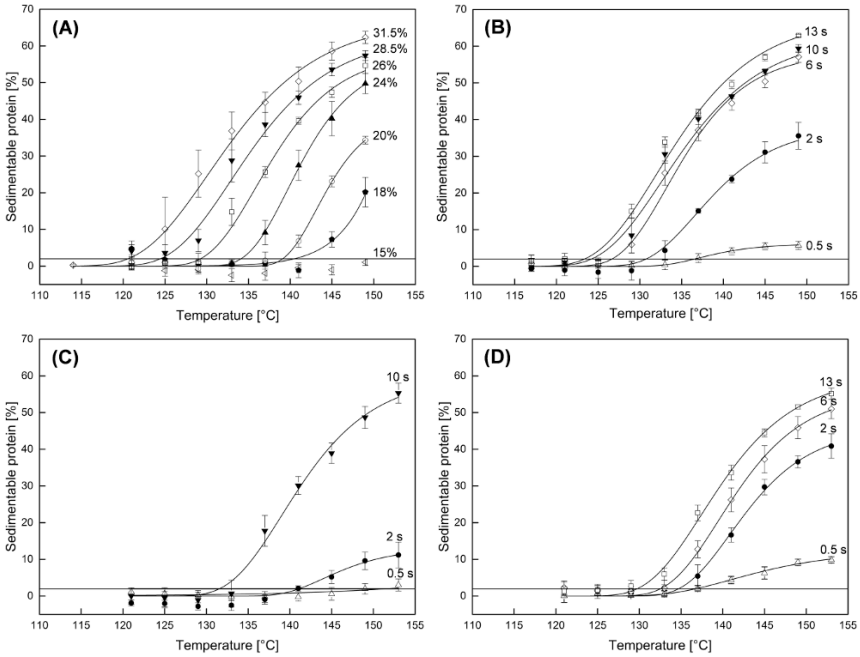

Fig. 4-7: Sedimentable aggregated protein of concentrated skim milk of different total solids at 9.5 s holding time (A), at different holding times at constant total solids content of 27% (B), 23% (C), and CSM of 27 % total solids made from UHT preheated milk (D). The onset of coagulation is indicated by a threshold level of 2% aggregated protein of total protein. Data points were fitted by a Gompertz model.

Tab. 4.2: Parameters of the Gompertz equation fitted to the data of sedimentable protein after direct steam injection heat treatment of CSM of various total solids content.[a]

TS_0 (%)	TS_T[a]	$S_{p,max}$	Gompertz parameters		$T_{crit,9.5s}$ (°C)[a]
			T_c	k	
31.5	30.3	67.3	130.1	0.1323	120.6
28.5	27.3	63.1	132.9	0.1470	124.4
26	24.7	58.3	135.9	0.1833	129.3
24	22.6	56.7	139.7	0.2162	134.1
20	18.7	41.8	143.1	0.2761	139.1

[a]Abbreviations are: TS_0, initial total solids; TS_T, actual total solids content of the CSM in the holding tube due to dilution by condensate; $S_{p,max}$, maximum sedimentable protein; $T_{crit,9.5s}$, critical temperature of 2 % sediment.

Fig. 4-8 shows the temperature-time combinations (black dots) leading to 2% of sediment for different total solids levels as well as different holding times on a semi-logarithmic scale as a mean of two replicates. In Fig. 4-8 we used a z-value of 10.6 as mean of the z-values described in Tab. 4–3 for interpolation. The total solids contents indicated were corrected for dilution by condensate. Condensate formation within CSM reduces the actual total solids content in the holding tube where heat-induced coagulation takes place. This can be seen as a further advantage of this technique for heat treatment of CSM irrespective of the fast heating and cooling to avoid heat-induced aggregation or to extend the temperature-time range to higher temperatures or holding times compared to indirect heat treatment.

Tab. 4–3: Parameters of the Gompertz equation of different holding times at 27, 23% total solids, and at 27% totals solids of UHT preheated milk.

Holding time (s)	$S_{p,max}$	T_c	k	T_{crit} (°C)a	*z*-value (K)
27%TS					
13	67.8	132.0	0.1469	123.4	9.33
10	62.9	132.6	0.1472	124.2	
6	58.4	133.2	0.1856	126.7	
2	38.1	136.7	0.1829	130.7	
0.5	6.2	137.0	0.2465	136.6	
23%TS					
10	59.9	139.0	0.1640	131.6	11.9
2	13.0	144.1	0.2144	141.1	
0.5	-	-	-	147b	
27%TS preheated					
13	60.2	137.4	0.1597	137b	
6	55.8	139.5	0.1737	135b	
2	45.8	140.9	0.1859	133b	
0.5	12.2	141.0	0.1397	130b	

[a]T_{crit} is the critical temperature for 2% of sedimentable protein; values with an asterisk are from visual determination. The *z*-value describes the increase in temperature necessary to reduce the time necessary to induce coagulation by 90%.

In this diagram, we suppose that we actually plotted the transition from the first step of coagulation, i.e. dissociation reactions, to heat-induced aggregation. The aggregation reaction itself was shown to have an activation energy of about 100-150 kJ mol^{-1} (White and Sweetsur 1977; Darling 1980). Interestingly, the *z*-values we derived from our data are close to the values obtained for the inactivation of bacterial spores. This, of course, means that there is no compromise between the onset of heat-induced coagulation if we use 2% sedimentable protein as a threshold and the inactivation of bacterial spores.

Effect of preheating on heat stability of skim milk concentrate

In Fig. 4-8 we also indicated the effect of preheat-treatment on the onset of coagulation depending on holding time at a constant total solids content of 27 % which was then corrected for dilution by condensate (squares). It can be seen that preheat-treatment shifts the heat stability to higher temperatures or longer holding times as expected from studies on the effect of preheat treatment on heat stability for evaporated milk manufacture under commercial sterilisation conditions (Newstead et al. 1979; Pearce 1979; Singh and Tokley 1990). As an example of a preheat treatment of unconcentrated milk, this UHT preheat treatment was chosen. Concentrates prepared from UHT milk also showed increased heat stability in terms of critical temperature-time conditions using the lab scale heat stability test of Dumpler and Kulozik (2015). In addition, this heat treatment offers the opportunity of sterilisation of milk without coagulation as well as sufficient enzyme inactivation that might be impossible due to coagulation of CSM when suitable heat treatments were applied to CSM. Indeed, conventional preheating at 115-120 °C for 1 min was more effective at 27% total solids on lab scale and may prove to be even more effective to increase heat stability of CSM (data not shown).

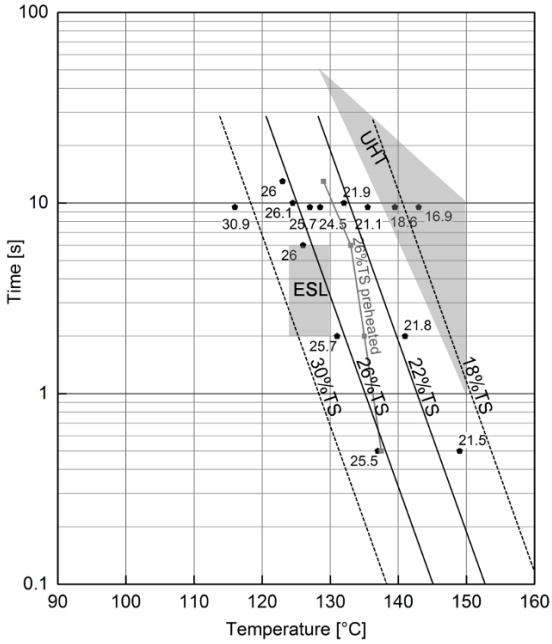

Fig. 4-8: Iso-effect lines of the onset of coagulation (2% of sedimentable protein of total protein) of concentrated skim milk heated by direct steam injection plotted on a semi-logarithmic scale. The 2% level of sediment for forearmed milk is indicated. The working areas used for ESL and UHT treatment of unconcentrated milk are shown for orientation.

To our knowledge, there is no data published on the effect of preheat treatment on the increase in heat stability of CSM within the UHT area as well as heat stability of CSM in this area in general. However, the knowledge that UHT heat treatment is possible for evaporated milk or reconstituted evaporated milk of 18-22% non-fat total solids has long been established in industrial practice (Muir 1984; Smith and Malmgren 1999). From the data obtained in this study, we also concluded that UHT heat treatment of non-preheated CSM is possible up to a total solids content of 18%. An appropriate heat treatment will result in an increase in heat stability in a way that evaporated milk of about 20% total solids will be UHT sterilisable as is common practice in industry. UHT preheating of skim milk in an increase in heat stability of CSM, whereby at very short holding times we could not observe a difference in the onset of coagulation between preheated and non-preheated concentrates. Sediment formation was even more pronounced at 0.5 s holding time compared to non-preheated concentrated milk as shown in Fig. 4-7 (D) compared to Fig. 4-7 (B). A difference in the dissociation kinetics of denatured associated whey protein-κ-casein complexes could be a reason.

4.4 Conclusion

Novel insights in the area of heat stability of concentrated milk on pilot scale are presented. This field of dairy research has been of interest for researcher for more than a century now. The extent of heat-induced aggregation of RO concentrated skim milk was shown to be dependent on skim milk concentrate total solids as well as the temperature-time combination chosen for heat treatment by direct steam injection. Iso-effect lines for a range of total solids content of CSM were derived enabling process design at a practical level. By adjustment of the parameters for DSI heat treatment below the onset of heat-induced coagulation, no sediment formation will occur. The differences in heat stability in terms of temperature-time combinations of CSM concentrated by evaporation or reconstituted concentrated skim milk depending on total solids as well as other preheating conditions need to be addressed. These differences could be determined by the heat-stability method presented in this study and would thereby further facilitate successful continuous in-line processing of concentrated milk products. Different kinds of milks, compositional differences as well as changes in mineral equilibrium due to temperature differences during concentration or removal of urea by RO concentration of milk might be possible reasons for differences in overall heat stability of differently processed milk concentrates. The quantitative determination of these differences in terms of maximum temperature-time relationships to maximize microbial inactivation will lead to shelf stable and safe milk concentrates either for powder manufacture or as an energy efficient liquid milk product. Protein aggregation and subsequent sediment formation can also be limited by holding times below 1 s and sterilisation of skim milk concentrates is likely to be possible when small amounts of sediment can be tolerated. Preheat-treatment of uncon-

centrated milk prior to concentration led to an increase in heat stability. An immediate applicability of the results on industrial scale is possible. A transfer of the results to continuous indirect heat treatment considering heating up and cooling down phases as contributing to heat-induced coagulation is likely.

Summary and contribution of the doctoral candidate

Continuous UHT processing for heat treatment of milk has largely replaced in-container sterilisation in the long life dairy category. However, for heat treatment of concentrated milk systems, in-container sterilisation is still very common as heat-induced product changes that result in browning and flavour development are desired and the sterilizability of the concentrate can be predicted by a conventional heat stability test. This test simulates the batch sterilisation process on lab scale where samples are heated, e.g. in an oil-bath at the sterilisation temperature until visual coagulation occurs. The temperature-time profiles and holding temperature of the standard lab scale test are rather similar to commercial sterilisation regimes. The concentrate is sufficiently heat stable when the coagulation times are longer than the time required for commercial sterility. Nevertheless, continuous UHT heat treatment of concentrated milk is becoming increasingly important due to several economic and environmental reasons. In contrast to in-container sterilisation, there is no data available on heat stability of concentrated milk under UHT conditions at high temperature and short holding times and an experimental setup to derive stability criteria. In addition, mechanistic aspects of coagulation under these conditions were merely described in literature.

Hence, the aim of this study was to describe heat stability in terms of maximum temperature-time combinations and heat-induced coagulation of concentrated skim milk using a pilot scale continuous steam injection system. Untreated and UHT preheated skim milk should be used to manufacture concentrated skim milk by RO.

This study showed that the relationship between heating temperature, heating time, and total solids content of the concentrates that had been established on lab scale was also valid for continuous heat treatment. However, maximum temperature-time combinations before the onset of coagulation and the slope of the regression for lines of equal effect were different from lab scale experiments due to differences in temperature-time profiles of the two systems. A preheat treatment of skim milk prior to concentration resulted in a shift of the onset of coagulation to higher temperature-time combinations. Particle size distribution analysis as well as electron micrographs of the unheated and heat treated concentrates showed that coagulation of casein micelles results in two particle fractions, dissociated casein particles and large aggregates. The composition of these fractions needed to be further addressed.

The substantial contributions of the doctoral candidate to this study were the design of the steam injection system, the supervision of its construction and successful implementation for the pilot trials performed for this study. The pilot trials were

done by the doctoral candidate together with technicians and students. Prior to writing major parts of this manuscript, literature had been critically reviewed, data had been collected, evaluated, calculations were carried out, and data had been plotted and included in tables and figures.

5 Dissociation and coagulation of caseins and whey proteins[8]

Abstract

Heat treatment of concentrated milk systems for preservation and long shelf life or at least a sufficient removal of food pathogens prior to spray drying is a crucial step due to the decreasing stability of these systems towards heat compared to unconcentrated milk. Heat-induced coagulation is observed when temperature-time combinations for the achievement of certain microbial inactivation effects are higher than the heat and colloidal stability of the concentrated milk system allows. In this work, the effects of direct steam injection on the stability of casein micelles in concentrated skim milk (CSM) of 18, 23 and 27% total non-fat solids, heat treated by direct steam injection (DSI), were investigated. Quantitative differential centrifugation for the separation of aggregates, casein micelles, dissociated submicellar particles, and soluble proteins, and subsequent analysis of caseins and whey proteins within these fractions by RP-HPLC were applied. Quantitative separation was monitored by particle size measurements. The dissociation of κ-casein as well as an increase in casein micelle hydrodynamic radius was observed to increase with increasing total solids content of CSM, heat treatment time, and temperature. Heat-induced dissociation of β-lg-κ-casein complexes at a critical level of 30-35% was found to induce severe coagulation of κ-casein depleted calcium-sensitive casein micelles in CSM heated by DSI. Dissociated and aggregated proteins were found to be present as distinct colloidal particle classes differing in size from casein micelles.

8 Original publication: Dumpler J, Wohlschläger H, Kulozik U (2017) Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. Dairy Sci & Technol 96:807-826. doi:10.1007/s13594-016-0304-3. Adapted original manuscript. Adaptions of the manuscript refer to enumeration type, citation style, spelling, notation of units, and format. Permission for the reuse of the article is granted by Springer International Publishing AG.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_5

5.1 Introduction

Concentrated skim milk (CSM) prepared by evaporation or reverse osmosis is manufactured as an intermediate product for spray-dried powders or long-life dairy products such as evaporated milk in today's dairy industry. Prior to spray drying, total solids concentrations of 40-50% are desirable due to the high energy consumption of the drying process per kilogram of water removed compared to evaporation. Interestingly, the structure and integrity of casein micelles remains unaltered in concentrated milk systems and the effects on micelle hydration, charge and colloidal calcium phosphate are reversible upon dilution depending on temperature and time of storage. Viscosity changes were attributed to an increase in the volume fraction of casein micelles which can be considered as highly hydrated spherical microgel particles (Olivares et al. 2016). High heat treatment of highly concentrated milks is relatively unusual to date due to immediate spray drying for preservation, low heat stability, and high fouling potential. Nevertheless, concentrated milk with 18-22% total non-fat solids is usually sterilized either in containers or these days more frequently continuously by heat exchangers. Problems associated with evaporated milk manufacture are usually reported as coagulation of evaporated milk during heat treatment, sediment formation, and age gelation during storage (Muir 1984; Smith and Malmgren 1999; Hinrichs 2000). Heat stability, i.e. the ability of concentrated milk systems to withstand a certain heat load, has been investigated for many decades in the context of sterilised evaporated milk. Mostly, these instabilities of concentrated milk towards heat treatment are referred to the casein micelles and their decreasing stability in a low pH, high ionic strength, and especially high soluble calcium environment (Sievanen et al. 2008; On-Nom et al. 2012; Crowley et al. 2014; Crowley et al. 2015).

Heat-induced coagulation of overall protein was taken as a means for characterization of changes in concentrated milk either by visual determination or by automated methods. The effects of serum composition, technological treatments, and total solids on the onset of coagulation of milk and concentrated milk have been extensively characterized on a lab scale in batch heating systems of small amounts of sample (White and Davies 1958; Rose 1961a, 1962; White and Davies 1966; Morrissey 1969; Fox and Hearn 1978a; Muir et al. 1979; Fox 1981; Kelly and Roefs 1990; Huppertz 2014). Dissociation of κ-casein together with associated β-lactoglobulin as well as chemical reactions are considered as the main destabilising effects on casein micelles leading to aggregation (Singh and Fox 1985a, 1986, 1987a, 1987c; van Boekel et al. 1989a; Singh and Latham 1993). However, further physical reactions preceding coagulation, their extent required to induce coagulation, and the kinetics of these elementary steps, especially in continuous heating systems like direct steam injection heat treatment, have been studied to a much lesser extent. Some studies have reported on heat-induced dissociation of κ-casein from casein micelles in milk and concentrated milk on a laboratory scale (Anema and Klostermeyer 1997; Anema 1998; Anema and Li 2000). However, a clear correlation between heat-induced coagulation and

the dissociation of caseins and whey proteins from micelles was not investigated to our knowledge.

In a previous publication, we found that there is a strong relationship between the total solids content as well as temperature and duration of a heat treatment on the onset of protein aggregation in CSM which had first been established on a lab scale (Dumpler and Kulozik 2015). Using direct steam injection, we could show that chemical reactions can be considered as unnecessary for heat-induced coagulation of CSM (Dumpler and Kulozik 2016). Particle size measurement revealed that for the induction of coagulation, native casein micelles first increase in size and during coagulation, disintegration of more and more micelles leads to small particles <100 nm and the formation of large proteinaceous particles in the range of 3-100 μm. However, the composition of the large particles and small submicellar particles, the reason for the rapid increase in sedimentable protein as well as the involvement of whey proteins in the coagulation process of CSM heated by DSI was still uncertain.

Therefore, in this study, we further addressed possibilities to quantitatively separate and analyse all the different protein fractions by differential centrifugation and subsequent analysis by a RP-HPLC method according to Bobe et al. (1998).

The determination of the distribution of caseins and whey proteins in the coagulated and dissociated fraction was intended to give mechanistic insights into the determinants of the onset and progression of the coagulation process of CSM.

5.2 Materials and methods

Concentrate manufacture and heat treatments

Pasteurised skim milk was obtained from a local dairy (Molkerei Weihenstephan, Freising, Germany). The average main composition of the skim milk and preparation of concentrates by reverse osmosis were as described by Dumpler and Kulozik (2015). Heat treatments of CSM of 18, 23 and 27% total solids at different temperatures and holding times using pilot scale DSI were performed as described previously.

Centrifugal separation of protein fractions in heat treated concentrated skim milk

The procedure for the separation of the different particle fractions is summarized in Fig. 5-1. Centrifugation of heat treated concentrated skim milk at 4,000*x*g for 10 min using a laboratory centrifuge (Multifuge 1S-R, Heraeus Holding GmbH, Hanau, Germany) was used to separate colloidal soluble casein particles from aggregated casein particles (fraction 1). The supernatant was then used for RP-HPLC analysis. The composition of the small particles (fraction 3) was determined by ultracentrifugation of the heat treated concentrated skim milk for removal of colloidal stable casein micelles (fraction 2) from the supernatant. Five hundred microliters of centrifugal supernatant was mixed with 900 μL of distilled water in a 1.5 mL Ultra Microtube (Thermo Scientific, NC, USA), properly mixed and centrifuged at 48,800*x*g for 26 min

at 25 °C using a Thermo Scientific Sorvall WX 80+ ultracentrifuge equipped with a Fiberlite F50L-24x1.5 rotor (Thermo Electron LED GmbH, Langenselbold, Germany). Therefrom, 900 µL of supernatant containing soluble protein and small casein micelle particles was withdrawn and used for further analysis.

For complete removal of colloidal protein and the determination of soluble protein (fraction 4), heat treated CSM was diluted approximately 1:3 (w/w) and approximately 30 g were filled into the centrifuge tubes. Ultracentrifugation was performed at 70,000*x*g for 60 min at 25 °C using a Beckman Coulter L7-65 equipped with a Ti-70 rotor (Beckman Coulter GmbH, Krefeld, Germany). One hundred microliters was pipetted from the centrifuge tubes by carefully immersing the pipette tip through the thin lipid phase on top into the serum phase.

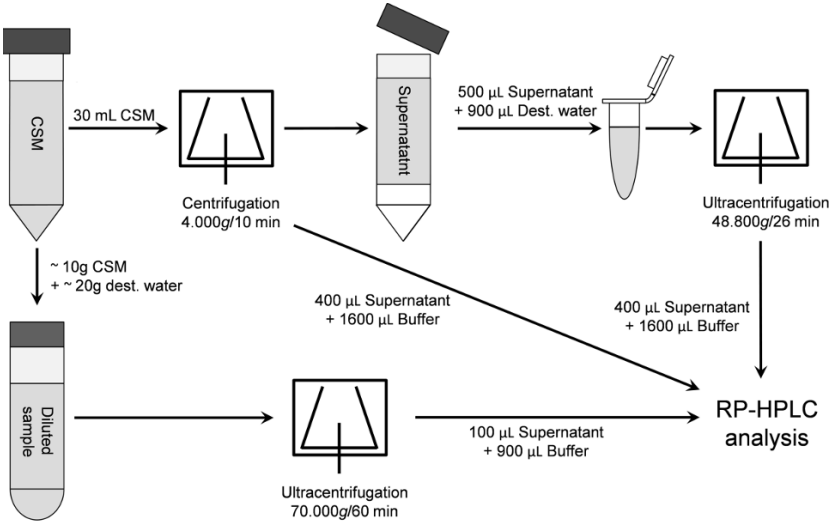

Fig. 5-1: Sample preparation procedure for separation of the different particle fractions and RP-HPLC analysis.

To summarize, this procedure aimed at separating four different classes of particles depending on their particle size and sedimentability.

Fraction 1: Aggregated protein (3-100 µm) sedimented at 4,000*x*g/10 min
Fraction 2: Colloidal stable micelles (100-1000 nm) sedimented at 48,800*x*g/26 min
Fraction 3: Submicellar particles (20-100 nm) sedimented at 70,000*x*g/60 min
Fraction 4: Soluble protein (< 20 nm), non-sedimentable

RP-HPLC analysis of major soluble proteins in concentrated skim milk
Supernatants containing individual fractions were dissolved in guanidine buffer for denaturation and dissolution according to Bobe et al. (1998) with some modifications

for simplification of the sample preparation procedure, shorter run times and reproducibility. This method needed some improvements according to concerns raised by the authors. Therefore, the major objectives concerning this method development were

- Modification and optimization of this method for the analysis of CSM,
- Sample stability at room temperature,
- Simplicity of sample preparation,
- No carry over between individual runs,
- Proper resolution as well as quantification of caseins and whey proteins in one single run

Attribution of different genotypes of caseins to the resulting spectrum of peaks was done according to Bonfatti et al. (2008). Compared to Bobe et al. (1998), we used five times the amount of sodium citrate (21.5 mM) to ascertain that colloidal calcium phosphate gets completely dissolved even in concentrated milk and to have an additional buffering capacity. Thereby, the final pH of the buffer solution was 7.2. In contrast to Bonfatti et al. (2008) reporting instability of the milk proteins, especially the whey proteins when dissolved in guanidine buffer and left at room temperature for several hours, we diluted milk, supernatant, or concentrated milk in the buffer in a ratio of 1:5. This results in a higher final guanidine concentration (5.1 M) in the prepared samples. Doing so, we could not observe a decrease in the amount of whey proteins even after storage of the sample plus buffer solutions for 72 h at room temperature (data not shown). This decay in detectable amount of whey proteins in the samples of Bonfatti et al. (2008) is likely to have occurred due to the incomplete denaturation of whey proteins or subsequent refolding at lower guanidine concentrations as investigated by Greene and Pace (1974). This buffer solution was also proven to be superior to a urea buffer solution as described by Bonizzi et al. (2009) for our purpose as it allows separating and quantifying caseins and whey proteins in one single run and gives a better resolution of individual caseins. Analytical standards of individual caseins and whey proteins were purchased from Sigma-Aldrich, Steinheim, Germany.

As a sample preparation procedure 400 µL of concentrated milk or supernatant was dissolved in 1600 µL of guanidine buffer as a single-step sample preparation and left for at least 30 min at room temperature for complete dissolution. The mixture was then filtered through a 0.45 µm regenerated cellulose syringe filter into a screw-capped glass vial. In the case of ultracentrifugal supernatant treated at 70,000*x*g for 60 min, 100 µL was dissolved in 900 µL buffer solution and treated similarly.

Analysis was performed on an Agilent 1100 Series chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump. The system was controlled by Agilent ChemStation software (Rev. B.04.03). Separation was performed on a C18 analytical silica-based column (Agilent Zorbax 300SB-C18, 4.6 x 150 mm, 5 µm). An analytical guard cartridge (Agilent Zorbax 300SB-C18, 4.6 x 12.5 mm, 5 µm) was used.

Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in 90% HPLC grade water and 10% acetonitrile. Solvent B was 0.07% TFA in 10% HPLC grade water and 90% acetonitrile. The flowrate was 1.2 mL min^{-1}, the column temperature was kept constant at 40 °C and detection was made at a wavelength of 220 nm. Injection volume was chosen between 20 and 80 μL depending on the expected concentration of proteins within the sample. The following gradient was used for separation: linear from 27 to 32% in 2 min, 32 to 38% B in 8 min, 38% B for 3 min, from 38 to 40% B in 3 min, from 40% B to 42% in 4 min, from 42 to 44% B in 2 min, 44% B for 0.5 min, then rinsing with 100% A for 4.5 min and 100% B for 4 min and returning to 27% B within 0.5 min. The total time of each run was therefore 31 min. Coefficients of variation for a five times sample preparation (repeatability) were 0.52, 2.25, 2.16, 1.96, 2.01, 1.03, and 1.04% for κ-casein, α_{s2}-casein, α_{s1}-casein, β-casein, α-lactalbumin, β-lactoglobulin B, and β-lactoglobulin A, respectively. Coefficients of variation were slightly higher for heat treated milk samples. Four subsequent 80 μL injections of 6 M guanidine buffer ('blank injection') at a runtime of 2 min each at 100% solvent A were used as a cleaning procedure. A subsequent shortened blank run of 7 min with 80 μL of guanidine buffer blank injection was used for cleaning the pre-column and column if carry-over of α_{s1}-casein and β-casein was detected.

The relative amount of caseins and whey proteins was calculated from the relative peak areas of heat treated and centrifugally separated CSM, i.e. the supernatant fraction, compared to the unheated centrifuged samples. Heat treatment of milk and concentrated milk lead to the formation of pre-peaks relative to the main whey protein peak of α-lactalbumin, β-lactoglobulin B, and β-lactoglobulin A. Standard deviations were calculated from two individual samples of each heat treatment that was performed in duplicate (n = 4). Data was plotted and statistical analysis was performed using OriginPro 2015G (OriginLab Corporation, Northampton, MA, USA). A paired sample *t*-test was used to test for significance between treatments and the amounts of individual proteins in the supernatant fractions.

Particle size analysis

Particle size measurements of CSM heat treated by direct steam injection and uncentrifuged and centrifuged at 4,000*x*g for 10 min were performed on a Malvern Mastersizer 2000 equipped with a Malvern Hydro 2000S sample dispersion unit (Malvern Instruments GmbH, Herrenberg, Germany) as described in an earlier publication (Dumpler and Kulozik 2016). Each sample was measured in duplicate at 20°C. Results are reported as the mean of two trials including measurements of two individual samples.

Supernatants of the centrifugation at 4,000*x*g for 10 min were diluted 1:50 in simulated milk ultrafiltrate (SMUF) according to Jenness and Koops (1962) and measured by photon correlation spectroscopy (PCS) in a Malvern Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany) as described previously. Supernatants of the centrifugation at 48,800*x*g for 26 min were diluted 1:5 with SMUF. Results of the particle size measurements reported in this study were calculated as means of eight measurements of two individual samples and two individual trials. Data were trans-

formed into a logarithmic density distribution q_3 [ln(x)] for better interpretability of the bimodal distributions observed.

5.3 Results and discussion

Particle size distributions in heat treated and fractionated concentrated skim milk

The measurement of particle size and particle size classes in CSM was performed due to two different assumptions made based on visual observations. First, we observed a rather white appearance and a higher viscosity of CSM after heat treatment which indicated that whey proteins have denatured upon heating and casein micelles had increased in size during heat treatment. Second, we could see a relatively rapid formation of sediment in samples that had coagulated even without acceleration of the sedimentation process by centrifugation. This indicated that large protein microparticles were formed due to the heat treatment. The sample preparation procedure based on these observations aimed to quantitatively fractionate the different particle classes. This was checked by the resulting particle size data. RP-HPLC was then used to determine their protein composition either directly or indirectly by analysis of the protein composition the supernatant fractions.

Fig. 5-2a, b shows the particle size distribution of CSM after heat treatment determined by laser light diffraction based on Mie theory (Mastersizer 2000) of the whole CSM ample as well as the supernatant of CSM centrifuged at 4,000*x*g for 10 min. Fig. 5-2c, d shows the results of the PCS measurements of the supernatants of 4,000*x*g for 10 min and the ultracentrifugal supernatant of 48,800*x*g for 26 min. The two different instruments were used as the whole range of particle sizes over four decades of size within the heat treated CSM samples could only be covered by using two instruments. The supernatant fraction of 4,000*x*g/10 min was found not only to contain non-aggregated casein micelles, but with increasing temperature, there was a marked increase in particles in the range of 20-100 nm. These particles could not be detected by laser light diffraction measurements based on Mie theory as the instrument had a detection limit of 40 nm.

It can be seen from the graphs (Fig. 5-2a, b) that centrifugation conditions at 4,000*x*g for 10 min resulted in a complete removal of large aggregates so that we could assume that the determination of proteins in the supernatant by RP-HPLC would give us quantitative information about the relative amount of individual aggregated caseins. In addition to this, we could observe a marked increase of the hydrodynamic radius of the casein micelles. This increase in hydrodynamic radius was dependent on both total solids content and holding time during heat treatment which will be discussed in more detail further down. O'Connell and Fox (2000) have shown that the velocity of increase is higher for larger micelles compared to smaller ones. This can be used to explain the broadening of the size distribution observed in this study prior to coagulum formation as shown in Fig. 5-2b, c. The faster increase in hydrodynamic radius of lager micelles due to dissociation within casein micelles

compared to smaller ones might explain a broader size distribution of casein micelles after a certain heat treatment.

In a second step, we additionally measured the particle size in this supernatant fraction by PCS for the detection of even smaller particles. We could observe that when aggregation of casein micelles occurs, a smaller fraction of particles in the range of 20-100 nm becomes a significant proportion of the distribution (Fig. 5-2c) whereby non-aggregated micelles become larger in size as also observed in laser light diffraction measurements (Fig. 5-2b). The particle size range of this small particle fraction was independent of temperature, holding time, and total solids content of CSM.

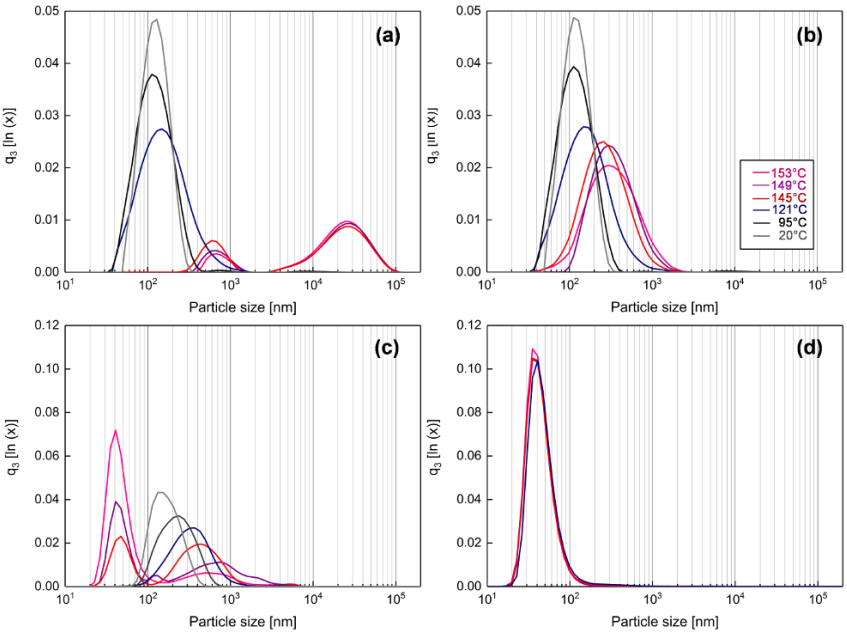

Fig. 5-2: Logarithmic density distributions of CSM heat treated at different temperatures at 23% total solids for a constant holding time of 10 s and subjected to differential centrifugation. Panels **a**, **b** were obtained by using laser light diffraction based on Mie theory and panels **c**, **d** were obtained by PCS. **a** Density distribution of the whole sample, **b** of the supernatant fraction (4,000*x*g/10 min), **c** of the supernatant fraction (4,000*x*g/10 min) and of the supernatant fraction (48,800g/26 min).

However, the distribution of fractions only gave quantitative information about the relative distribution of these fractions within a certain sample. To isolate this fraction from the heat treated CSM samples, to analyse its composition, and to study its quantitative evolution at different heating temperatures and holding times, we developed an ultracentrifugation protocol for complete removal of quasi-native casein micelles.

At the same time, we wanted to ensure that these smaller submicellar particles remain quantitatively in the supernatant. We therefore determined the minimally required ultracentrifugation conditions for the removal of casein micelles from the samples. The required intensity of the centrifugal conditions for the removal of casein micelles was much lower compared to conditions used in literature (Carroll et al. 1971; Singh and Fox 1985a, 1987a; Singh and Creamer 1991). This might be partly explained by considering that centrifugation conditions also depend on the size of the centrifuge tubes used which were comparably small (1.4 mL) in our study. The particle size distribution of these isolated particles is shown in Fig. 5-2d. A selection is shown for temperatures where these particles of 20-100 nm in size were present in a large quantity. Quality criteria for PCS measurements were not met for ultracentrifugal supernatants at lower temperatures, indicating a too small amount of particles present in the ultracentrifugal supernatants and thus a complete removal of casein micelles which would give a strong signal even at low concentration. Carroll et al. (1971) also observed small particles in concentrated milks by electron microscopy and suggested that it is not only composed of denatured whey protein particles, but rather composed of 'small micellar units' as they called it.

A dilution step prior to ultracentrifugation was introduced to minimize interactions between particles during sedimentation. In addition, dilution of the samples was a necessary step to reduce the volume of the pellet and its significance on the results obtained. Concerns about the implications of the dilution with distilled water on casein micelles might be raised. However, total CSM solids content of minimum 18% was diluted by a dilution factor of 2.8 for ultracentrifugation at 48,800*x*g/26 min which means that the minimum ionic strength was 67% of the original unconcentrated milk. The same procedure was used for all total solids contents which resulted in a relative ionic strength of 85.5% and 100% for 23 and 27% total solids, respectively. For ultracentrifugation at 70,000*x*g/60 min, the dilution factor was about 3 as shown in Fig. 5-1. Ultracentrifugation was performed immediately after dilution within 1 h. The insignificance of the dilution step was proven by our RP-HPLC results of unheated samples presented later in this study. These did not differ for different dilutions of the unheated samples.

Fig. 5-2 was one example for the different fractions of particles in CSM at 23% total solids after heat treatment. To further investigate the effects of total solids, temperature, and time of heat treatment on casein micelle size using DSI, the changes in particle size should be related to heat-induced dissociation of protein material from casein micelles and micelle aggregation. Fig. 5-3 shows the volume based average particle diameter $d_{50,3}$ of casein micelles supernatants in heated CSM. It can be observed that in general, the mean diameter of casein micelles increases with increasing heating temperature and holding time at a constant total solids content (Fig. 5-3a) and increases with increasing total solids content at a constant temperature-time combination (Fig. 5-3b) for 18 and 27% total solids. Samples of CSM with 23% total solids and 10 s holding time were also presented as data for a holding time of 6 s were not investigated. From these results, we concluded that preheating to 95°C without direct

steam injection did not largely affect casein micelle size in the range of total solids content investigated. At higher temperatures, there is a steady increase of casein micelle size and up to a more than twofold increase in size at a maximum. These observations should be born in mind as we will refer these observations to protein dissociation and heat-induced aggregation.

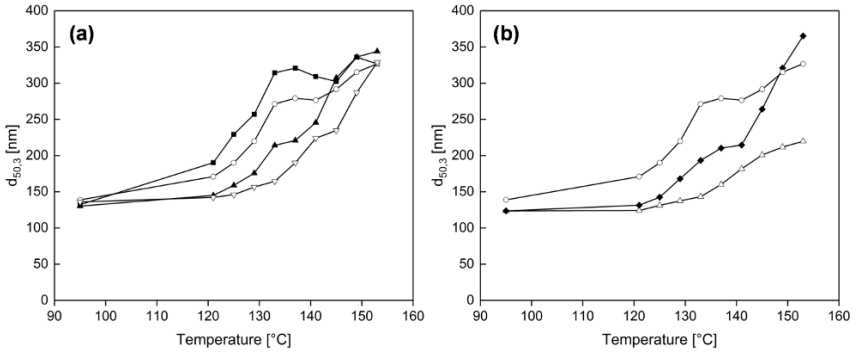

Fig. 5-3: Average volume-based particle diameter $d_{50,3}$ as a function of temperature, total solids, and holding time of DSI heat treatments. **a** Shows holding times of 0.5 s *(open triangles)*, 2 s *(closed triangles)*, 6 s *(open circles)*, and 13 s holding time *(closed squares)* at 27% total solids of the supernatant (4,000*x*g/10 min) of CSM samples. Figure **b** shows the average volume based particle size $d_{50,3}$ as a function of total solids for 18%TS/6 s *(open triangles)*, 23%TS/10 s *(closed diamonds)*, and 27%TS/6 s *(open circles)*.

Heat-induced aggregation of caseins in concentrated skim milk

To follow the aggregation process in CSM at different heating temperatures which led to distinct and separable large particles, we used RP-HPLC to separate the individual caseins and whey proteins remaining in the supernatant fraction in a first step. Fig. 5-4 shows three different chromatograms of caseins and whey proteins as an example indicating the aggregation of α_S-caseins and β-casein and also some whey protein with increasing heating temperature. The dilution of the sample with guanidine buffer gave a better resolution of individual proteins compared to dissolution of milk samples in urea buffer as used by Rauh et al. (2014), Bonizzi et al. (2009), and Visser et al. (1991). In the case of whey proteins, higher heat intensity leads to a pre-peak formation due to non-enzymatic glycosylation of whey proteins with lactose as described by (Rauh et al. 2015). By using this method, we could quantitatively follow the heat-induced aggregation process of individual caseins at heated different temperatures and heat holding times at constant non-fat total solids content of 27 % as presented in Fig. 5-5. It was observed that calcium-sensitive α_S-caseins and β-casein start to become sedimentable due to aggregation when a critical temperature-time combination is exceeded. The amount of aggregated β-casein was significantly lower ($p < 0.05$) compared to α_{S1}-casein for samples where aggregated protein was present. It is interesting to note at this point that samples were completely stable

up to a certain critical temperature. Exceeding this critical temperature led to a rapid increase of coagulated sedimentable protein.

In contrast to this, κ-casein content decreased with increasing holding time to a much lesser extent and there was no marked difference between holding times. These observations are in agreement with the results of Mora-Gutierrez et al. (1993) concerning the calcium sensitivity and tendency of self-association of individual caseins when we consider heat-induced coagulation as a heat-induced re-arrangement process. Interestingly, the tendency of heat-induced aggregation of the individual caseins in the order

$$\alpha_{S1} \geq \alpha_{S2} > \beta >> \kappa$$

was contrary to the dissociation behaviour of individual caseins from casein micelles in milk heated at high temperature observed by Anema and Klostermeyer (1997), Singh and Creamer (1991), and Holt et al. (1986). The α_S-caseins in milk are known to be the most calcium-sensitive caseins due to their phosphoserine-containing polypeptide chain (Holt et al. 2013). These observations may also be related to the position of the caseins within the casein micelle which fosters the dissociation of caseins from the outer surface rather than the core regions of the micelle.

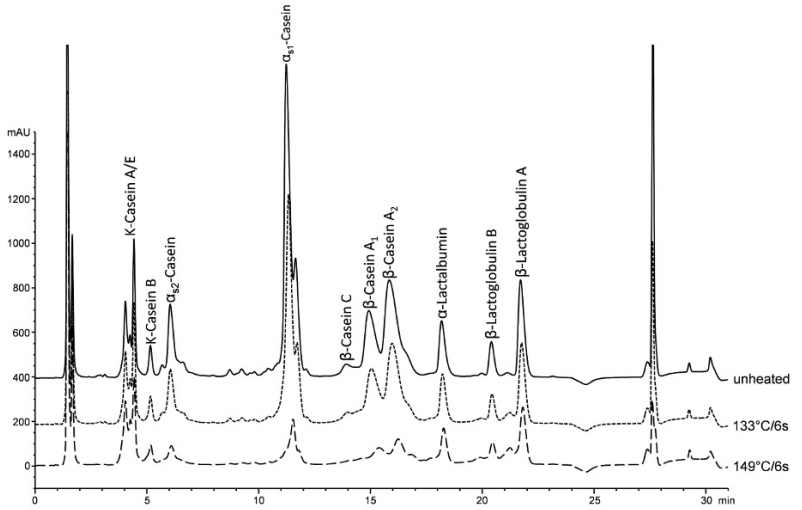

Fig. 5-4: RP-HPLC chromatograms of supernatants (4,000*x*g/10 min) of caseins and whey proteins in guanidine buffer. Three samples are shown indicating the reduction of calcium sensitive caseins in the supernatant.

The κ- and β-caseins are also known to be more loosely bound to the casein micelle by hydrophobic interactions (Dalgleish and Corredig 2012a; Holt et al. 2013). Dissociated caseins were not incorporated into large aggregates. This could be a result of

the self-association of κ- and β-caseins into stable soluble complexes together with whey proteins in the size range of 20-100 nm (fraction 3). A slight decrease in the amount of non-sedimentable κ-casein might be perceivable in Fig. 5-5 due to incomplete dissociation and co-aggregation with calcium-sensitive caseins but could also be a result of thermal degradation. There was an indication for thermal degradation of caseins in the HPLC chromatograms as peaks of individual caseins were changing their shape.

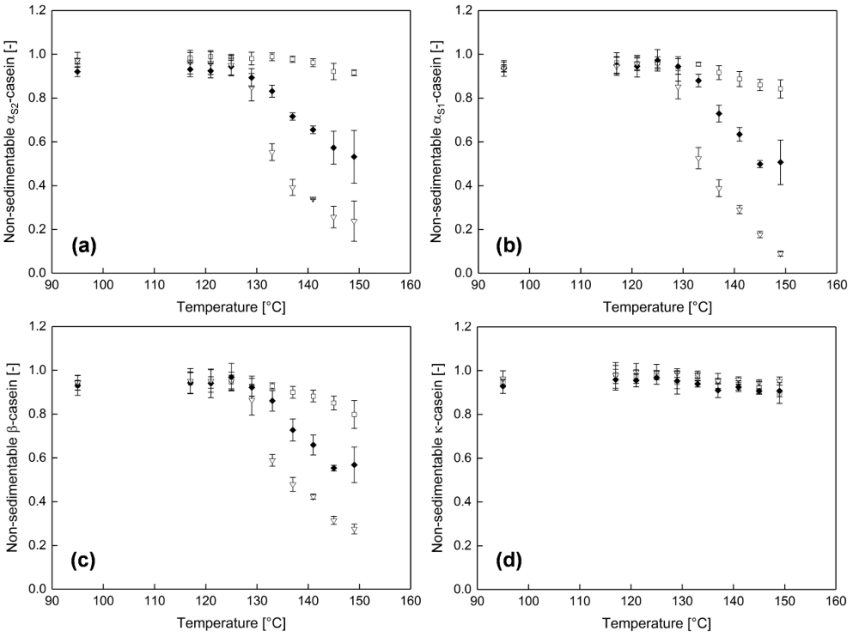

Fig. 5-5: Relative amount of non-sedimentable caseins in CSM at 27% total solids centrifuged at 4,000*x*g for 10 min heated at different temperatures and holding times of 0.5 s *(squares)*, 2 s *(diamonds)*, 10 s *(triangles)*. **a** α_{s2}-casein, **b** α_{s1}-casein, **c** β-casein, **d** κ-casein.

These results indicated that heat-induced coagulation and the subsequent sedimentability of protein are mainly a result of the heat-induced aggregation of κ-casein-depleted calcium-sensitive micelles. The calcium-sensitive caseins in the core of casein micelles contribute to most of the protein present in CSM as determined as sedimentable protein at high temperatures in a previous publication (Dumpler and Kulozik 2016). Therefore, this aggregation process as second step must be a temperature-time-dependent reaction that is limited by a preceding rate-determining reaction of κ-casein dissociation. Considering the results of particle size measurements, we suggest that the dissociated material does not form soluble proteins but tends to form new quasi-micellar structures that we observed in supernatants of strongly coagulated samples as presented in Fig. 5-2c, d.

Critical level of casein dissociation to induce coagulation

Following this hypothesis, we investigated centrifugation conditions that quantitatively separated these small particles from quasi-native casein micelles. Soluble proteins like undenatured whey proteins and soluble caseins are also included in this fraction. Examples for the chromatograms of differently heat treated CSM of 27% total solids centrifuged at 48,800*x*g for 26 min is shown in Fig. 5-6. It can be seen from the chromatograms that mainly the amount of κ-casein increased significantly with increasing intensity of heat treatment whereby the amount of calcium-sensitive casein increased to a lesser extent.

Fig. 5-7 shows the amount of non-sedimentable caseins in the centrifugal supernatant obtained at 4,000*x*g for 10 min *(closed symbols)* and the ultracentrifugal supernatant from samples centrifuged at 48,800*x*g for 26 min *(open symbols)*. Three different CSMs with varying total solids content of 18, 23, and 27% total solids were investigated. Holding time was 6, 10, and 6 s, respectively. The graphs show that the onset of coagulation, indicated by the rapid decrease of non-sedimentable calcium sensitive caseins, as well as the extent of coagulation are both dependent on CSM total solids as well as holding time at a certain temperature. The reduction of the relative amount of κ-casein was seen to be also unaffected of CSM total solids and did not coagulate to a large extent together with calcium-sensitive caseins. The coagulation of β-casein was again less compared to α_{S1}-casein. It is therefore most likely that κ-casein-depleted calcium-sensitive micelles aggregate as spherical protein particles to form large sedimentable particles.

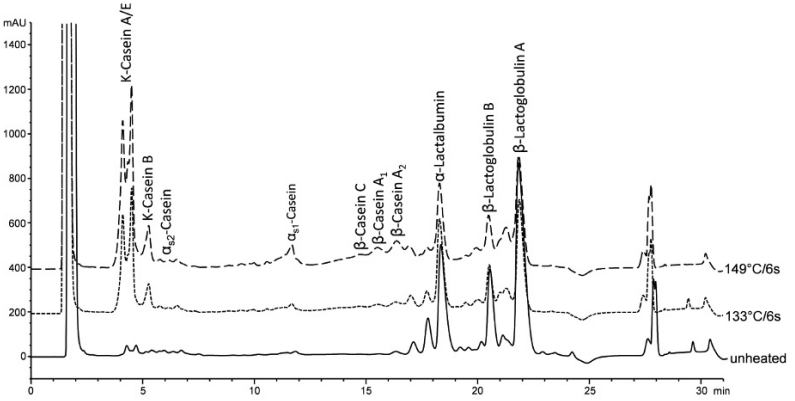

Fig. 5-6: RP-HPLC chromatograms of supernatants (48,800*x*g/26 min) of caseins and whey proteins in guanidine buffer. Three samples are shown indicating the increase in the amount κ-casein in the supernatant with increasing heating temperature.

The dissociation of individual caseins was inversely related to the tendency of caseins towards aggregation. There was a very limited dissociation of calcium-sensitive α_S-caseins. The apparent presence of a certain amount of α_{S2}-casein in the serum

phase is likely to be the result of peptides underlying the peak of a_{S2}-casein. Considering this, the extent of heat-induced β-casein dissociation (Fig. 5-7c) was significantly higher ($p < 0.01$) compared to α_S-caseins at constant total solids content. The extent of κ-casein dissociation was found to increase linearly with temperature and total solids content of CSM to a large extent as shown in Fig. 5-7d *(open symbols)*. At the highest temperature applied, between 60 up to 80% of κ-casein had dissociated from the micelles into fraction 3 (small submicellar particles) at 18 and 27% total solids, respectively. At the same time between 50 and up to 80% of calcium sensitive casein had aggregated to large sedimentable particles (fraction 1). The effect of total solids on the integration of calcium sensitive caseins into fraction 3 was not significantly different ($p < 0.05$).

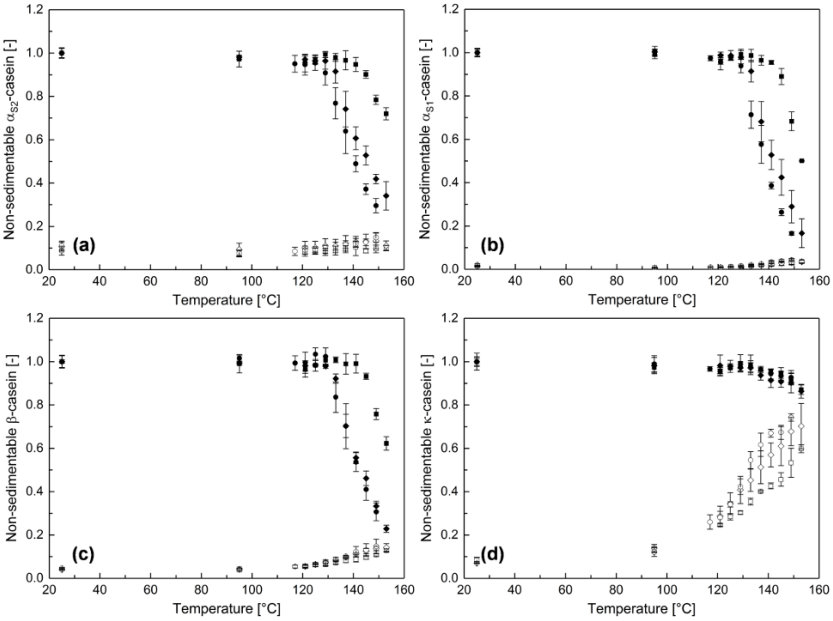

Fig. 5-7: Relative amount of non-sedimentable caseins in CSM at 18%/6 s holding time *(squares)*, 23% totals solids/10 s *(diamonds)*, and 27% total solids/6 s (*circles*) centrifuged at 4,000*x*g for 10 min *(closed symbols)* and 48,800*x*g/26 min *(open symbols)* heated at different temperatures. **a** α_{S2}-casein, **b** α_{S1}-casein, **c** β-casein, **d** κ-casein.

The results indicate that κ-casein dissociation limits the aggregation of calcium-sensitive caseins during the coagulation process. In addition, we assumed that individual micelles having higher κ-casein content relative to the amount of calcium-sensitive caseins, i.e. especially smaller casein micelles, are less prone to dissociation and aggregation. This was also proposed and investigated by O'Connell and Fox (2000) and Aoki and Kako (1983). To address this hypothesis, the relative distribution

of caseins in fraction 2 (quasi-native micelles) was calculated from the difference in the amounts present in fraction 1 (large particles) and dissociated and soluble proteins (fraction 3 and 4) using the data presented in Fig. 5-7. As a result, we found that the relative amount of κ-casein based on the total amount of caseins in the fraction of non-aggregated quasi-native micelles (fraction 2) was decreasing due to dissociation of κ-casein up to the critical temperature of coagulation at 18, 23, and 27% of total solids. With further increasing temperature, at the point where a large proportion of calcium-sensitive caseins starts to aggregate, the proportion of κ-casein within fraction 2 is increasing again, indicating that smaller, κ-casein-rich micelles are more resistant towards heat-induced coagulation. Most interestingly, the critical relative amount of κ-casein dissociation at the onset of coagulation in CSM heat treated by direct steam injection was in the range of 30 to 35% taking into account 8% of dissociated κ-casein in the unheated CSM.

The observations that κ-casein does not aggregate together with calcium-sensitive caseins, that there is a critical level of κ-casein dissociation that is likely to induce coagulation, and that the κ-casein to calcium sensitive-casein ratio within the population of non-aggregated micelles increases, led us to the following conclusion. There is a distribution within the population of micelles in terms of κ-casein depletion that tends towards aggregation. Completely depleted micelles become present at a certain temperature and holding time and this population grows faster under more unfavourable conditions, i.e. higher total solids content. The effect of heat-induced dissociation and aggregation needs to be further addressed.

Effect of temperature and heating time on aggregation and dissociation of α-lactalbumin and β-lactoglobulin

Whey proteins are known to interact with κ-casein either during heat treatment of milk or concentrated milk. Structures that form during heat treatment can be very variable due to the changing reactivity of the free thiol group of β-lactoglobulin (β-lg) at different pH values. This interaction was found to lead to soluble β-lactoglobulin-κ-casein complexes when heat-induced dissociation of κ-casein from casein micelles occurs. In another proposal for the formation of these complexes, it was suggested that dissociated κ-casein forms disulphide linkages in the serum phase and the subsequent re-association of κ-casein is hampered and thereby reduces the stability of the casein micelles. α-Lactalbumin (α-la) is known to form complexes with β-lg during heat treatment, and it was shown that α-la-β-lg-κ-casein complexes are possible in the presence of β-lg in milk (Anema and Li 2003b; Donato and Guyomarc'h 2009). Hence, the observed dissociation of κ-casein and subsequent aggregation of the calcium sensitive caseins should lead to a redistribution of whey proteins as well. Fig. 5-8 shows the distribution of the whey proteins α-la and β-lg in the fractions ultracentrifugal supernatant (open symbols) and the large sedimentable aggregates (closed symbols). The fraction of casein micelles and particles in this size range is again represented by the residual amount. About 10% of α-la was not soluble in CSM (Fig. 5-8a). This may be due to pasteurization of the milk prior to concentration. Additional 20% of α-la got associated with the casein micelles during preheating to

95 °C without steam injection. The degree of denaturation of α-la was about 30-35% after preheating so that more than more than half of the denatured α-la got associated with casein micelles and therefore sedimentable. The degree of denaturation was determined as described by Dumpler and Kulozik (2016). However, no differentiation between undenatured α-la and small colloidal complexes with β-lg could be made. At higher temperatures, α-la dissociates again from the micelles to some extent, the dissociation being more pronounced at higher total solids content of CSM. Above 140 °C, there might be an aggregation into larger particles and also some thermal degradation of α-la as the relative amount of α-la decreases in both fractions 1 and 3.

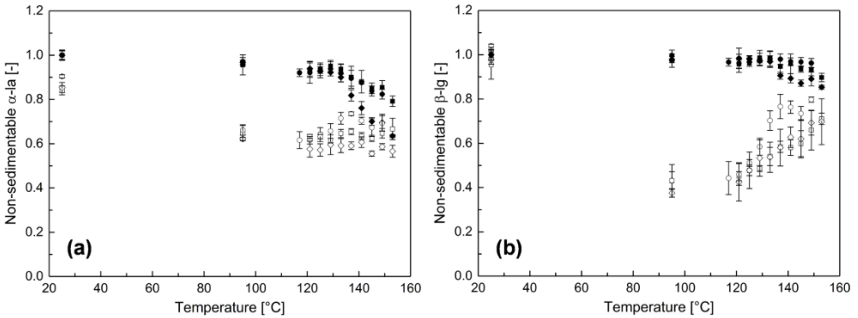

Fig. 5-8: Relative amount of non-sedimentable whey proteins in CSM at 18%/6 s holding time *(squares)*, 23% totals solids/10 s *(diamonds)*, and 27% total solids/6 s (*circles*) centrifuged at 4,000*x*g for 10 min *(closed symbols)*, and 48,800*x*g/26 min *(open symbols)* heated at different temperatures. **a** α-lactalbumin, **b** β-lactoglobulin.

Fig. 5-8b shows that β-lg association with the casein micelles is more strongly pronounced compared to α-la when CSM is preheated to 95°C. About 60% of β-lg associates with casein micelles and become sedimentable by ultracentrifugation. Increasing the heating temperature resulted in a dissociation of β-lg from casein micelles that resembles the behaviour of κ-casein, and therefore, we assume that β-lg-κ-casein complexes dissociate from the micelles including some associated α-la. Again, the dissociation of β-lg was more pronounced when the total solids content of CSM was higher at a constant temperature. Sedimentation of β-lg together with calcium-sensitive caseins was very limited. Whey proteins may therefore exist in four different forms distributed in each fraction in CSM when heat-induced aggregation occurs. These different forms can be denoted in descending order of their corresponding amount: dissociated whey protein-κ-casein complexes, soluble denatured whey protein complexes, native whey proteins, and co-sedimenting whey protein-casein aggregates.

Amount of soluble protein formed during heat treatment of concentrated skim milk

Fig. 5-9 shows the amount of soluble β-casein and κ-casein in the ultracentrifugal supernatant obtained after centrifugation at 70,000*x*g for 60 min depending on hold-

ing time and temperature of CSM with 27% total solids. α_S-Caseins could not be detected in the ultracentrifugal supernatant at 70,000*x*g for 60 min. By comparison of Fig. 5-9a, b, it turns out that the amount of soluble κ-casein is independent of the aggregation of calcium-sensitive caseins and rather follows the dissociation of κ-casein-β-lg complexes from the micelle surface as shown in Fig. 5-7d. The amount of κ-casein rather increases linearly with temperature and was found to be not significantly higher ($p > 0.1$) when the holding time was longer. However, at least means were higher at longer holding times. The amount of soluble κ-casein (Fig. 5-9b) divided by the amount of κ-casein dissociated from casein micelles (Fig. 5-7d) is in the range of 6 to 8% at all temperatures. The overall amount of soluble β-casein and was found to be not significantly different between heat treatments in terms of holding time and total solids ($p > 0.1$) and was at all temperatures less than 1.5% of total β-casein.

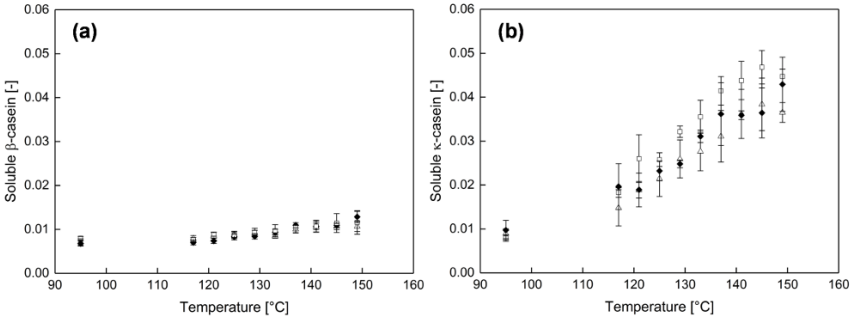

Fig. 5-9: Relative amount of **a** soluble β–casein and **b** soluble κ-casein in the ultracentrifugal supernatant (70,000*x*g/60 min) after heat treatment of concentrated skim milk of 27% total solids at different temperatures for 2 s *(triangles)*, 6 s *(diamonds)*, and 13 s *(squares)*.

Soluble protein was therefore found to be a minor fraction that cannot explain the onset and propagation of coagulation of casein micelles. When we consider the small particles and the amount of dissociated κ-casein found within this fraction, we can conclude that for a sedimentation of 70% of caseins in sum (27%TS; 149 °C/6 s), 80% of kappa-caseins need to be dissociated from the micelle (Fig. 7d, 149 °C/6 s). Thereby, we could close the mass balance of protein detected in different fractions. We concluded that the material dissociated from the micelles due to heat treatment of CSM is mainly incorporated in small colloidal quasi-micellar particles of 20-100 nm in diameter (fraction 3) as observed by PCS particle size measurements. These particles mainly consist of κ-casein-whey protein complexes including comparably small amounts of calcium-sensitive caseins. Proteins dissociated from the micelles were not found to be present as soluble protein after a certain amount of heat load to a large extent.

5.4 Conclusions

To conclude on the effects of heat treatment by direct steam injection on concentrated skim milk leading to heat-induced coagulation with special focus on casein micelles and whey proteins, we drew a schematic representation of the steps involved that could be derived from the analytical results. This model is shown in Fig. 5-10. It describes the conversion of a native, colloidally stable micelle into a dissociated form as a first step. Due to the activation energy supplied by heat, the micelle will dissociate into κ-casein-whey protein complexes and calcium-sensitive κ-casein-depleted casein micelles. These micelles are increasingly loose in structure, much larger, and stronger hydrated compared to the native structure possibly due to the cleavage of the linkage between caseins and colloidal calcium phosphate as described by Aoki et al. (1990). This massive increase in hydrodynamic radius may induce or at least facilitate κ-casein dissociation and may increase attractive forces due to a much larger calcium-sensitive 'sticky' contact area depleted in κ-casein and therefore increase the rate of aggregation into large protein particles that are sedimentable.

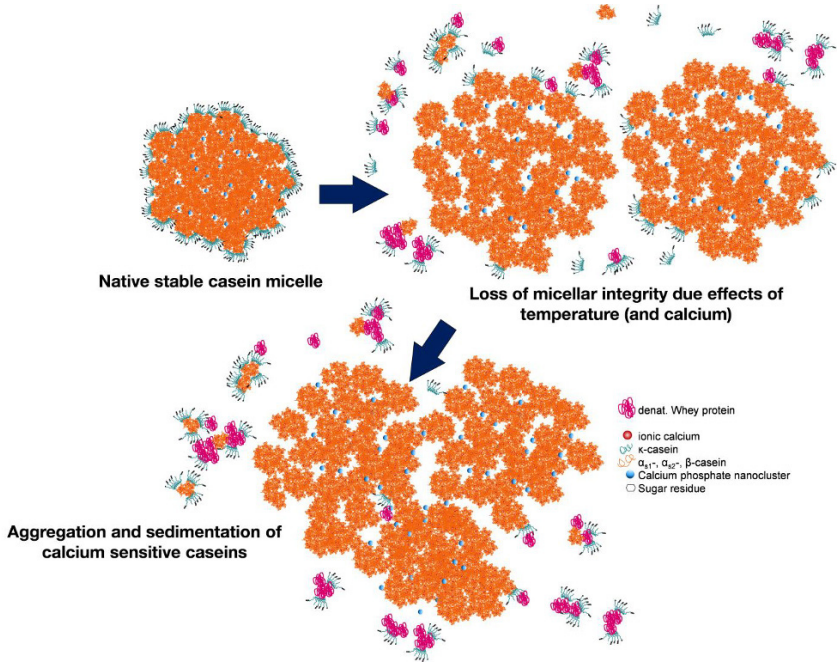

Fig. 5-10: Schematic representation of the proposed model of heat-induced dissociation and coagulation of caseins and whey proteins in CSM as derived from the distribution analysis of individual proteins.

The observed effects of the increase in casein micelle size and the dissociation of κ-casein-whey protein complexes from the casein micelle surface were found to parallel each other as these effects both increased with increasing total solids content as well as heating temperature and time. Therefore, a close relationship between loosening of the internal micellar structure and protein dissociation leading to destabilisation of casein micelles is likely. The effects of serum composition, pH, and volume fraction of casein micelles on the velocity of κ-casein dissociation and especially the increase in hydrodynamic radius of casein micelles need to be further addressed. Crowley et al. (2015) have shown that an increase in ionic calcium increased the rate of κ-casein dissociation.

Most relevant in this work was the observation that heat-dissociated proteins were not found as soluble in the serum phase but rather forms submicellar colloidal particles together with denatured whey protein that can be separated by ultracentrifugation. Ultracentrifugation conditions that removed all colloidal particles from milk used in literature might have underestimated the extent of dissociation of casein from the micelles during heat treatment. In addition, we could gain new insights into the second undesired step of heat-induced coagulation which is the aggregation process leading to a redistribution of caseins into different size fractions and the formation of new structures when a direct high heat process is applied for preservation of liquid CSM for the reduction of the environmental impact of the dairy industry by using CSM as a skim milk powder substitute.

Summary and contribution of the doctoral candidate

Heat stability is generally defined as the stability of milk to withstand heat treatments without visible coagulation. The tendency of milk towards the formation of a coagulum and subsequent sediment formation during storage is dependent on milieu conditions which change during concentration. However, little knowledge exists on physico-chemical reactions that induce destabilisation of casein micelles in CSM and therefore precede coagulation, especially under pilot scale and commercial in-line sterilisation systems.

Therefore, the aim of this study was to investigate the effect of direct steam injection on dissociation and coagulation of caseins and whey proteins in CSM. The fractions of heat treated CSM, i.e. the different particle classes, obtained by a differential centrifugation protocol from heat treated CSM, were intended to give insights into the mechanism of coagulation of concentrated skim milk under commercial sterilisation conditions. Centrifugal and ultracentrifugal supernatants were analysed by reversed phase high performance liquid chromatography (RP-HPLC). A buffer for solubilisation of proteins in milk and concentrated milk and subsequent analysis by RP-HPLC was adapted from literature and amended to separate and quantify all major milk proteins in one single run within the fractions. Quantitative separation of the

different fractions by centrifugation and changes in casein micelle size were monitored by particle size measurements.

The results of this study showed that upon heating, κ-casein dissociates from casein micelles together with denatured whey proteins. Dissociated caseins were observed to form distinct submicellar particles and to a much lesser extent soluble caseins. Each fraction could be quantitatively separated in the ultracentrifugal supernatants. Calcium sensitive caseins were found to dissociate to a much lesser extent. The κ-casein depleted micelles, containing mostly calcium sensitive caseins, were found to coagulate into large aggregates. Whey proteins were found in all fractions depending on temperature-time combinations applied. Exceeding a critical level of 30-35% of dissociated κ-casein and an approximately twofold increase in the average hydrodynamic radius of casein micelles, severe coagulation of concentrated milk of 18, 23, and 27% total solids was induced. The increase in micelle size upon heating increased with increasing total solids content of CSM and temperature which paralleled the tendency of heat-induced coagulation and indicated a correlation. A schematic representation of the process was drawn.

The substantial contributions of the doctoral candidate were the critical review of literature concerning the RP-HPLC analysis of caseins and whey proteins, solubilisation of milk proteins in buffer solutions, and ultracentrifugal fractionation of casein micelles. The method development was assisted by buffer development, sample preparation, design of experiments for validation, and the adaption of the method to different milk systems. The doctoral candidate developed the differential centrifugation method, performed the pilot scale heat treatment trials, and carried out data analysis and evaluation. The manuscript was to the most extent written by the doctoral candidate.

6 Preparation of simulated milk ultrafiltrate[9]

Abstract

In milk, the serum phase is in a dynamic equilibrium with insoluble salts within the casein micelle and therefore saturated with respect to calcium phosphate depending on pH, temperature and other compositional factors. In this study, simulated milk ultrafiltrate (SMUF) was therefore considered as a dynamic medium that approximates ultrafiltration (UF) permeates of a certain milieu condition resulting in changes in calcium and phosphate content as detected by ion chromatography. Due to reported spontaneous precipitation of existing SMUF solutions and interference with measurements and experiments, SMUF solutions were developed according to the chemical composition and physical parameters of UF permeate at different temperatures. SMUF solutions were proven to be as stable as their corresponding ultrafiltration permeates as they were similar in composition and calcium activity. Calcium activity was considered as a useful parameter to estimate the onset of supersaturation and spontaneous precipitation depending on calcium concentration and pH.

9 Original publication: Dumpler J, Kieferle I, Wohlschläger H, Kulozik U (2016) Milk ultrafiltrate analysis by ion chromatography and calcium activity for SMUF preparation for different scientific purposes and prediction of its supersaturation. Int Dairy J 68:60–69. doi:10.1016/j.idairyj.2016.12.009. Adapted original manuscript. Adaptions of the manuscript refer to title, enumeration type, citation spelling, notation of units, and format. Permission for the reuse of the article is granted by Elsevier Limited.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_6

6.1 Introduction

Simulated milk ultrafiltrate (SMUF) is an aqueous salt solution that simulates the mineral composition of milk serum obtained by ultrafiltration (UF). Since the pioneering work of Jenness and Koops (1962), this solution has been widely used and was, in some cases, proven to precipitate spontaneously and therefore interfere with experiments. Jenness and Koops (1962) investigated the similarity between milk UF permeate and proposed SMUF solutions by selected chemical and physical parameters available at that time and have proven SMUF to be very close to UF permeate concerning these selected criteria. These criteria were ionic composition, freezing point, conductivity, pH, lactose content, total solids, buffering capacity, and heat-induced precipitation of calcium phosphate in milk permeate. They suggested it to be used for the dispersion of casein micelles obtained by ultracentrifugation to remove whey proteins from milk. They further suggested its use for removal of lactose from milk by dialysis against SMUF. This has successfully been carried out by using this buffer solution for studies on removing single constituents from milk to study their effect on colloidal and heat stability of modified milk systems (Fox and Hoynes 1975; Fox and Hearn 1978a; Singh and Fox 1987c; O'Connell and Fox 1999, 2001).

There were basically two major applications or categories of experiments SMUF has been used for. First, it was used to disperse biomolecules in synthetic SMUF for controlled processing or secondly, for analysis to preserve physico-chemical changes that had occurred due to processing. Hence, later studies have added multiple applications of SMUF like using it as a reactive medium for crystallisation studies of lactose or calcium phosphate in synthetic milk serum (Spanos et al. 2007), deposition of calcium phosphate on stainless steel surfaces or membranes depending on composition and hydrodynamics (Andritsos et al. 2002; Rosmaninho and Melo 2006), dilution of milk proteins for particle size measurements (O'Connell and Fox 2000; Beliciu and Moraru 2009), experiments to determine inactivation kinetics (Terebiznik et al. 2000; Bendicho et al. 2003) or to prove mathematical models on mineral equilibria in milk (Gao et al. 2010a; Gao et al. 2010b). Moreover, changes in the mineral equilibrium of milk serum due to changes in pH, temperature or of the calcium and phosphate concentration can be studied which will result in more or less precipitation of calcium phosphate.

These multiple applications are underlining the importance of simulated milk salt solutions in dairy research. Controlling reaction conditions by simulation as well as the simplification, standardization of conditions towards natural variation, and a reduction of complexity by composition of media is of outmost importance in research. To do so, modifications of the SMUF solution will be necessary as there have been reports in literature and observations in our laboratory that SMUF prepared by the recipe of Jenness and Koops (1962), especially the so-called dry blend mixture, is prone towards precipitation of calcium phosphate, often spontaneously, even when left at room temperature for a few hours. When used for diafiltration at elevated temperature or when the pH is adjusted to pH > 6.8, spontaneous precipitation was

observed at our lab. Physical analysis is made impossible as calcium phosphate crystals will interfere with particle sizing results. In addition, pH and composition of SMUF changes uncontrolled and often spontaneously (Beliciu and Moraru 2009). At ambient conditions, UF permeate obtained at 10 °C showed similar trends towards precipitation whereas UF permeate obtained at 50 °C was not prone to precipitation at pH values up to 6.8.

A possible solution for this problem is indicated in studies on milk ultrafiltration permeates at different temperatures. Rose and Tessier (1959), On-Nom et al. (2010), and Kaombe et al. (2012) have shown that filtration temperature in the range of 20 to 110 °C affects the pH as well as the amount of calcium and phosphate present in ultrafiltration permeates due to the formation of colloidal calcium phosphate bound to casein micelles. Calcium and phosphate are therefore retained in the retentate fraction during filtration. Higher filtration temperatures resulted in lower pH of UF permeates measurable at room temperature and lower amounts of calcium and phosphate present in the filtrate. These changes, induced by higher or lower filtration temperatures than room temperature, of course, will affect the mineral balance and the degree of supersaturation with respect to calcium phosphate when these permeates are used at room temperature.

In milk containing casein micelles, supersaturation of calcium phosphate due to an increase in temperature and pH will lead to precipitation of calcium phosphate onto casein micelles, i.e. the formation of colloidal calcium phosphate (van Dijk and Hersevoort 1992). Theoretical models on milk serum composition depending on pH exist, but have not yet included temperature as a crucial parameter (Gao et al. 2010a; Rice et al. 2010). Rice et al. (2010) found that citrate ions had a strong impact on calcium activity and on the solubility of calcium phosphate and they could determine the solubility limits for calcium in model solutions depending on the citrate concentration and other constituents. Despite these available modelling approaches, no adoption of the SMUF solutions towards the solubility limit for calcium phosphate depending on citrate concentration, pH and temperature based on empirical data were reported to date.

Hence, the aim of this study was to provide compositional data for both, i.e. mathematical models including temperature and for the preparation of SMUF solutions that are comparable to UF permeates at various working temperatures. The SMUF solutions of Jenness and Koops (1962) should be used as a starting point to extend the knowledge about properties and preparation of SMUF solutions. A second aim of the study was to predict the pH range of use for SMUF solutions without precipitation by comparative calcium activity measurements between SMUF and skim milk. Emphasis should be put on a simple and reliable method of preparation of SMUF solutions close to milk serum for multiple scientific purposes.

6.2 Materials and methods

Manufacture of ultrafiltration permeates

Pasteurised skim milk was obtained from a local dairy (Molkerei Weihenstephan, Freising, Germany). Ultrafiltration was performed on a pilot plant (Simatec GmbH, Schwalmtal, Germany) using a spiral wound UF membrane with a molecular weight cut-off of 10 kDa (GR70PE-6338/48; Alfa Laval Corporate AB, Lund, Sweden). A milk batch of 100 litres was used. Filtration was performed by recirculation of permeate. The feed flow was 18 $m^3 h^{-1}$ and an average transmembrane pressure of 2 bars was applied. Ultrafiltration permeates of skim milk of natural pH were taken at 10, 20, 30, 40, 50 and 55 °C. Filtration was started at 10 °C and temperature was risen after each sampling. For readjustment of the mineral equilibrium in milk, samples were taken 40 min after the temperature had been reached. However, subsequent analysis of samples that had been obtained after 0, 10, 20, 30, and 40 minutes at 10 °C resulted in no significant ($p > 0.05$) changes in ionic composition. The average of these four samples, analysed in triplicate, was taken for comparison of significant changes in composition with increasing temperature. Samples were immediately cooled to 4 °C and stored refrigerated until further analysis.

Sample preparation procedure for skim milk, ultrafiltration permeates, and SMUF for high performance ion exchange chromatography

Ion chromatography was used to determine the amounts of individual cations and anions in ultrafiltration permeates, SMUF, and skim milk. In dilute soluble systems like milk salt solutions, ion chromatography offers the opportunity to quantify all inorganic anions, and additionally, lactate and citrate in a single run (Gaucheron 2011). This is a major advantage compared to titration or photometric methods as well as other automated spectroscopic methods which are capable to determine single or a selection of ions or their atoms. In the case of cation chromatography, some non-protein nitrogen components (NPN) can also be detected, separated, and quantified.

Prior to analysis by ion chromatography, proteins need to be removed from milk protein containing systems. Strong chaotropic acids like perchloric acid will precipitate caseins together with whey proteins from milk as determined by RP-HPLC analysis of supernatants (data not shown). Colloidal calcium phosphate is solubilised and also ions bound to caseins are liberated quantitatively as pH of the solution drops to pH values lower than pH 2. Sample preparation was therefore as follows. 10 mL of milk were precipitated by addition of 500 µL 60% perchloric acid in a volumetric flask of 100 mL and shaken until complete coagulation of milk proteins. Afterwards, the flask was filled up to the calibration mark. Prefiltration of the suspension by a folded filter (Ø 150 mm; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and subsequent filtration through a 0.45 µm regenerated cellulose syringe filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was necessary for clarification of

the sample. Protein free UF permeates and SMUF solutions were diluted 1:10 and directly used for injection.

Determination of cations and anions in ultrafiltration permeates, SMUF, and skim milk by high performance ion exchange chromatography

All chemicals used were of analytical grade. Ion chromatography columns were integrated into an existing Agilent 1100 Series chromatograph equipped with a refractive index detector (Agilent Technologies, Waldbronn, Germany). The system was controlled by Agilent ChemStation software (Rev. B.04.03). Cation chromatography was performed using a HAMILTON PRP-X800 (4.1 x 250 mm, 7 µm) stainless steel column (Hamilton Bonaduz AG, Bonaduz, Switzerland) containing a PS-DVB resin functionalized with itaconate for cation exchange. Isocratic elution was performed using 2.3 mM oxalic acid, 0.4 mM sulfuric acid (pH 2.55) in HPLC-grade water for cations in milk serum-like systems. Flow rate was 1.0 mL min^{-1} and the oven temperature was set to 20 °C.

Calibration was performed using certified reference solutions for ion chromatography (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Linear calibration ranges were from 15 to 200 mg L^{-1} for sodium, ammonium, potassium, and calcium. Due to the small amounts of magnesium present in milk serum-like systems, calibration for magnesium was performed in the range from 13 to 50 mg L^{-1}. Coefficients of variation of a five times sample preparation of ultrafiltration permeate (50 °C) for sodium, potassium, magnesium, and calcium were 2.4, 1.9, 3.4, and 1.9%, respectively. Limit of detection (LOD) in diluted UF permeate samples as 3x the signal-to-noise ratio and limit of quantification (LOQ = 3 x LOD) were 1 and 3 mg L^{-1}, respectively.

Chromatograms of cations and anions in UF permeate and a SMUF solution is shown in Fig. 6-1. Fig. 6-1a and c shows the chromatogram obtained for cations in UF permeate and SMUF, respectively. Fig. 6-1b and d shows the chromatogram obtained for anions in UF permeate and SMUF, respectively. Low molecular weight non-protein nitrogen components (NPN) were also tentatively identified by retention times in Fig. 6-1a. Monoethanolamine, creatine, and creatinine could be detected as they become positively charged at low pH. Urea and ammonia were not completely separated from monoethanolamine under the conditions used (Fig. 6-1a).

Anion chromatography was done using a HAMILTON PRP-X100 (4.6 x 250 mm, 5 µm) PEEK column (Hamilton Bonaduz AG, Bonaduz, Switzerland) containing a strong anion exchange polystyrene resin functionalized with trimethyl ammonium groups. Isocratic elution was performed using 8 mM *p*-hydroxybenzoic acid, 0.83 mM Ca^{2+} (33 mg L^{-1}), 0.1 mM sodium thiocyanate in HPLC-grade water containing 2.5% methanol. A pH of 8.9 was adjusted with sodium hydroxide. Column oven temperature was set to 40 °C. Calibration ranges for anions were from 30 to 600 mg L^{-1} for carbonate, lactate, chloride, phosphate, nitrate, citrate, and sulphate.

Calibration was performed by using potassium salts of the anions. Calibration was checked by using certified standard solutions for the major ions chloride, phosphate and citrate (Fluka Chemie AG, Buchs, Switzerland). The injection volume for cations and anions was chosen in the range from 5 to 80 µL depending on the expected

amount present in the samples. Coefficients of variation of a five times sample preparation of ultrafiltration permeate (50 °C) for chloride, phosphate, citrate, and sulphate were 0.9, 1.9, 4.1, and 9.6%, respectively. LOD and LOQ in diluted UF permeate samples were 2.5 and 7.5 mg L^{-1}, respectively. UF permeate samples were diluted 1:10 with distilled water prior to injection.

Using a refractive index detector results in a lower sensitivity of the method compared to a more common conductivity detector for ion chromatography. However, there are advantages as well. As the mineral content of milk serum-like systems is expected to be high, less or no dilution is necessary. In addition, the determination of sugars and organic acids is possible using the same system by switching automatically to another column. Compared to a conductivity detector, there is no need for ion suppression prior to detection to minimize the conductivity of the eluent for a proper signal-to-noise ratio.

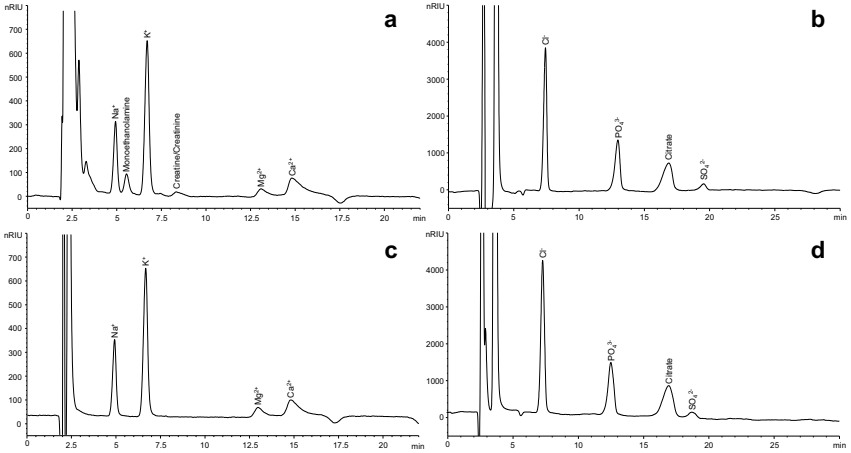

Fig. 6-1: High performance ion exchange chromatograms ultrafiltration permeate obtained at 50 °C (a and b) and simulated milk ultrafiltrate (c and d) for cations (a and c) and anions (b and d).

Determination of lactose in ultrafiltration permeates

Lactose was determined using the method as described by Schmitz-Schug (2014). According to her description, separation was performed on the HPLC system mentioned above using a BIO-RAD Aminex HPX-87H (300 x 7.8 mm, 9 µm) ion exclusion column filled with sulfonated divinyl benzene-styrene copolymer as a support. An Animex HPX-87H guard column was used. As isocratic eluent 5 mM sulfuric acid was used. Column oven temperature was set to 50 °C and flow rate was 0.6 mL min^{-1}. Protein free ultrafiltrates were diluted 1:10 for analysis.

Preparation of SMUF with different ionic composition

SMUF solutions according to Jenness and Koops (1962) were prepared as suggested with some modifications. Trisodium citrate dihydrate, magnesium hydroxide car-

bonate, and magnesium citrate nonahydrate were used for the preparation of the *wet* and *dry blend mixture*. Amounts to be added according to Jenness and Koops (1962) were recalculated according to the crystallisation water present. Therefore, 1.791 g and 0.656 g of trisodium citrate dihydrate and magnesium citrate nonahydrate, respectively, were used per litre of *dry blen*d mixture SMUF solution. In the case of the *wet mixture*, 17.91 g of trisodium citrate dihydrate and 3.89 g of magnesium hydroxide carbonate were added to stock solution I and II, respectively. The amount necessary to adjust the pH of 1 L solution to 6.6 was approximately 1.5 mL of 1 M KOH. An important modification of the method of Jenness and Koops (1962) was related to the addition of $CaCl_2$. Calcium chloride was added after the complete dissolution of the other salts of the *dry blend mixture* and not mixed with the other salts. Thereby, the formation of insoluble calcium phosphate crystals during dissolution was avoided. The non-uniform dissolution of the salts which cause shifts in pH and ionic composition during dissolution led to immediate precipitate formation in some cases.

As there were differences in composition of UF permeates and SMUF solutions of Jenness and Koops (1962) based on the results of the determination of the ionic composition of permeates presented in this study, alternative SMUF solutions were also prepared. The preparation procedure was simplified and precipitation of calcium phosphate during preparation of the SMUF was to be avoided. Individual salts and quantities required to simulate the composition of UF permeate obtained at 10 °C and 50 °C filtration temperature are summarized in Fig. 6-1. These quantities were obtained from an iterative Excel calculation procedure.

Tab. 6–1: Quantities of individual salts needed for the preparation of a simulated milk ultrafiltrate simulating ultrafiltration permeate at 10 °C and 50 °C.

Weight [g L^{-1}]			Amount [mM; SMUF 10 °C/SMUF 50 °C]							
SMUF 10 °C	SMUF 50 °C	Component	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	PO_4^{3-}	Citrate	SO_4^{2-}
0.933	0.833	KH_2PO_4		6.9/6.1				6.9/6.1		
0.867	0.753	K_2HPO_4		10.0/8.65				5.0/4.3		
0.7	0.533	$K_3C_6H_5O_7 \cdot H_2O$		4.9					2.1/1.6	
1.2		$Na_3C_6H_5O_7 \cdot 2\ H_2O$	12.2						4.0	
0.6		$Mg_3(C_6H_5O_7)_2 \cdot 9\ H_2O$				2.9			1.9	
0.057		$C_6H_8O_7 \cdot H_2O$							0.3	
0.633	0.933	KCl		8.5/12.5			8.5/12.5			
0.167		NaCl	2.9				2.9			
0.2		K_2SO_4		2.3						1.15
1.233	0.933	$CaCl_2 \cdot 2\ H_2O$			8.4/6.35		17.7/12.7			
0.112	0.056	KOH		2.0/0.7						
		Sum	15.1	34.5/35.2	8.4/6.34	2.9	29.0/28.1	11.9/10.5	8.4/7.9	1.15

Preparation of the SMUF solutions suggested in this study was simplified in comparison to the SMUF solutions suggested by Jenness and Koops (1962). All salts for the SMUF solution to simulate UF permeate at 10 °C (SMUF 10 °C) and 50 °C (SMUF 50 °C) filtration temperature, respectively, except calcium chloride were weighed into a weighing boat. These salts were then quantitatively transferred into a 1 L volumetric flask by rinsing with water. This solution can be prepared in a large quantity and can be frozen for storage and finished after thawing of the solution. Addition of calcium chloride and subsequent freezing will cause calcium phosphate precipitation and will make it unsuitable for physical analyses. After filling up the flask to approximately 75%, it takes about 5-10 min until complete dissolution of magnesium citrate. The flask is filled up to the calibration mark, the solution then transferred to a beaker and calcium chloride is added. The volume change by addition of calcium chloride is considered negligible. The pH of the solution is then adjusted to 6.75 for SMUF 10 °C and pH 6.65 for SMUF 50 °C by addition of 1 M potassium hydroxide. This should be done very slowly as titration at pH > 6.8 will cause calcium phosphate to precipitate from SMUF 10 °C and as the equilibration of SMUF after base addition takes time. These two solutions mainly differ with respect to the pH, phosphate and calcium level in the final solution. The addition of a certain amount of lactose is possible before the addition of calcium chloride into the flask or alternatively after the final pH adjustment by dissolution of lactose in a volumetric flask with SMUF solution. It was chosen in this study to add 49.5 g L^{-1} based on the results of the lactose determination in UF permeate.

Determination of the dry matter, freezing point, pH, and conductivity

Dry matter content of UF permeates and SMUF without and with added lactose was determined using a microwave dryer (CEM Smart turbo 5, CEM, Kamp-Lintfort, Germany). The freezing point was determined using a cryoscope (Cryo Star I, Funke – Dr. N. Gerber Labortechnik GmbH, Berlin, Germany). Two-point calibration was performed using standard solutions (freezing point of 0.000 and - 0.600°C according to EN ISO 5764:2009). The pH values, conductivity, and calcium activity were determined using suitable probes connected to a ALMEMO 2590-4AS (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany). The pH glass electrode was a HLBIGT-2 (LiBuTec GmbH & Co. KG, Langenfeld, Germany) connected to an adapter cable ZA9610-AKY4WR1E4 (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany). Conductivity was determined using a conductivity sensor FYA641LFP1 (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany). For its calibration, standards of 1.413 and 12.88 mS cm^{-1} (Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland) were used. Conductivity, pH value, calcium activity, freezing point and dry matter were determined at least in duplicate.

Determination of calcium activity using an ion selective electrode

Calcium activity in SMUF and UF permeate over pH was determined using a perfectION™ calcium selective combination electrode (Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland) connected to the ALMEMO device via a ZA9000 FS2 adapter cable (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany). The

pH of the samples was adjusted by dropwise addition of 1 M NaOH or HCl. Calibration was performed using 14 calcium standard solutions prepared from a 3.886 M standard solution ranging from 0.18 to 146.7 mM Ca^{2+} as calcium chloride. Calibration and measurements were performed at 25 °C. The potential in mV was recorded and plotted against the logarithm of the calcium activity. The measureable calcium activity $\alpha_{Ca^{2+}}$ in solutions is linked to the soluble calcium concentration $c_{Ca^{2+}}$ via the calcium activity coefficient γ_i

$$\alpha_{Ca^{2+}} = \gamma_i \cdot c_{Ca^{2+}} \tag{6.1}$$

Calcium activity was calculated from calcium concentration by calculating the activity coefficient γ_i depending on the ionic strength I in the standard calcium chloride solution using the empirical Davies equation (eq. 6.2) which is valid up to I < 500 mM. The factor A was set to 0.51 (Merkel and Planer-Friedrich 2008). z_i is the valency of the calcium ion.

$$\lg \gamma_i = - A \cdot z_i^2 \cdot \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3 \cdot I \right) \tag{6.2}$$

The ionic strength of a pure calcium chloride solution can be calculated using eq. 6.3

$$I = \frac{1}{2} \cdot \sum c_i \cdot z_i^2 \tag{6.3}$$

where c_i is the molar concentration of the cation and anions and z_i is the charge of the ion.

Calibration was performed every day immediately before the measurement of samples at 25 °C. Results of calcium activity were calculated from linear regression. The correlation coefficient of the linear regression was at least 0.994 in all cases. The slope of the electrode was on average 29.82 mV per decade of calcium activity difference varying from 29.28 to 30.37 mV at 25 °C. The reference slope according to the Nernst equation would be 29.58 mV.

In common practise, the ionic strength in the $CaCl_2$-containing calibration solutions is adjusted by addition of 70-100 mM NaCl or KCl or mixtures thereof to calibrate Ca^{2+} concentration instead of activity which results in some difficulties. By omitting this preparative step of adjustment of the ionic strength in calibration solutions, we avoided the imprecision resulting from salt addition, the type of salt used, and the differences in composition of calibration solutions and SMUF. From our experience, a compensation for the calcium selective electrode being not fully selective was not necessary as the potential of SMUF without added calcium was at least 30 mV (1 decade Ca^{2+} activity) lower than the lowest measured potential in SMUF containing calcium.

In this study, we used a direct linkage between measured potential in aqueous $CaCl_2$ solutions and the calculated calcium activity via the activity coefficient. The activity coefficient was estimated by the Davies equation (eq. 2). The measured po-

tential in SMUF solutions was then converted into activity by the calibration function. Nevertheless, an estimation of the soluble free calcium concentration based on activity is still possible from our data. This is because the calcium activity is directly linked to the measured potential in SMUF without estimation of the ionic strength in SMUF and UF permeates. As we adhere to primary data, the estimation of soluble calcium can easily be done by estimating an activity coefficient in SMUF by other analytical means and take it as a divisor. Therefore, this is a different approach to the problem of the estimation of free, soluble, and non-complex bound calcium. One of the major problems is that the ionic strength of milk salt systems cannot be calculated with sufficient precision. In literature, ionic strength of milk serum was reported to be in the range of 70-80 mM (de Kort et al. 2009; Gao et al. 2010a; Chandrapala et al. 2010; Gao et al. 2011; de Kort et al. 2012). In addition, a variation in calcium concentration for calibration in NaCl- and/or KCl-$CaCl_2$-systems results in a variation in the ionic composition that might influence the determined Ca^{2+}-activity. In the UF permeates as well as SMUF solutions, an ionic strength of 70 mM could be assumed. This would correspond to a calcium activity coefficient of 0.413 in $CaCl_2$-water systems and could be taken for approximate calculation of free soluble calcium. Gao et al. (2011) suggested a Ca^{2+}-activity coefficient of 0.37 for SMUF solutions determined by adopted calibration solutions and the determination of soluble calcium by other means.

Acid or base addition results in a change in the activity coefficient in SMUF, UF permeate, and skim milk due to a change in ionic strength. Changes were found to be negligible and within the experimental error. Calcium activity measurements were performed at least in triplicate.

Statistical analysis

A two sample t-test was used to compare the amounts of individual cations and anions in UF permeate for significant changes in composition with increasing temperature at a 95% significance level. The mean of four samples taken every 10 min at 10 °C over a period of 40 min during filtration and analysed in triplicate was used for comparison (n = 12) with samples obtained at higher temperatures. Variances were assumed to be non-equal. Statistical analysis was performed using OriginPro 2015G (OriginLab Corporation, Northampton, MA, USA).

6.3 Results and discussion

Ionic composition and physical properties of ultrafiltration permeate

Compositional analysis of UF permeates was used as a basis for the preparation of SMUF solutions. Results of the determination of cations and anions in UF permeate by ion chromatography in mg L^{-1} and mM are shown in Fig. 6-2. It can be seen that the results of the two individual filtration trials gave relatively similar results concerning the composition of UF permeates at different temperatures. Thereby, we in-

tended to show the repeatability of the filtration trial. Hence, results of the two trials were not averaged. Increasing the filtration temperature from 10 to 20 °C had no significant effect on UF permeate composition. At 30 °C, the amount of calcium and phosphate in UF permeates began to decrease. This was statistically significant ($p < 0.05$) in one out of two trials. At higher filtration temperatures, there was a significant decrease in the amount of calcium and phosphate present in permeates in both trials, while the amounts were decreasing with increasing filtration temperature.

Equal amounts at 10 and 20 °C might be due to two reasons. Firstly, there might be a hysteresis in the milk salt equilibrium resulting in a small supersaturation being possible as the milk was stored cold prior to filtration resulting in higher amounts of calcium and phosphate being soluble also at 20 °C. Secondly, analytical precision of the method might not have been sensitive enough to detect these minor changes from 10 to 20 °C. Interestingly, carbonate, as suggested by Jenness and Koops (1962) to be added to SMUF solutions, was not detected. This is also reasonable as carbonates will be expelled from their corresponding salts by stronger acids like citrate and phosphate being present in UF permeates. Carbonate addition will therefore just raise the pH as hydroxides would do.

Other constituents of UF permeate remained constant across all filtration temperatures. This indicates that the precipitation of calcium phosphate observed in the SMUF solutions as prepared according to Jenness and Koops (1962) at higher temperatures and pH was mainly due to too large quantities of calcium and phosphate present in solution compared to the saturation condition. Our results also confirm findings from the investigations of Rose and Tessier (1959) and Kaombe et al. (2012) who found that at more extreme temperatures of 70 and up to 110 °C, there were significantly lower amounts of soluble calcium and phosphate present in UF permeate from milk compared to room temperature. Their results at room temperature were found to be very close to the findings obtained in this study. Changes in calcium and phosphate content as well as pH as the first changes with increasing temperature can be attributed to the higher solubility of other poorly soluble salts of calcium and magnesium like sulphates and citrates compared to calcium phosphate. In the presence of casein micelles, calcium phosphate will precipitate onto casein micelles to a large extent (Hardy et al. 1984; van Dijk and Hersevoort 1992).

The stoichiometric calcium-to-phosphate ratio calculated as difference from UF permeates at 10 and 50 °C (Fig. 6-1) was found to be on average close to 1.5 which corresponds to the precipitation of amorphous calcium phosphate or more crystalline calcium deficient hydroxyapatite (Holt 1982; van Kemenade and de Bruyn 1987; Pouliot et al. 1991; Mekmene et al. 2012). Tab. 6–3 also includes other physical characteristics of the UF permeates.

Tab. 6–2: Ionic composition of ultrafiltration permeate obtained at different filtration temperatures.[a]

Analyte	Filtration temperature [°C]											
	10 °C		20 °C		30 °C		40 °C		50 °C		55°C	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Amount [mg L^{-1}]												
Sodium	382 ± 13	359 ± 4	375 ± 15	362 ± 8	388 ± 4	367 ± 4	364 ± 21	362 ± 1	364 ± 16	371 ± 9	385 ± 8	368 ± 4
Potassium	1392 ± 43	1447 ± 8	1406 ± 45	1467 ± 27	1406 ± 73	1474 ± 7	1342 ± 92	1452 ± 8	1405 ± 69	1493 ± 29	1444 ± 35	1464 ± 8
Magnesium	76 ± 4	86 ± 2	73 ± 3	86 ± 5	74 ± 1	89 ± 1	70 ± 2	88 ± 1	69 ± 3	89 ± 3	72 ± 1	83 ± 1
Calcium	345 ± 22	348 ± 7	346 ± 9	345 ± 17	324 ± 23	333* ± 5	279* ± 16	296* ± 8	266* ± 19	290* ± 6	274* ± 11	232* ± 4
Chloride	1009 ± 6	926 ± 9	976 ± 18	934 ± 14	1002 ± 9	934 ± 16	927 ± 57	919 ± 2	974 ± 55	938 ± 15	995 ± 17	925 ± 7
Phosphate	1163 ± 9	1161 ± 14	1135 ± 55	1147 ± 30	1066* ± 37	1122 ± 33	1005 ± 77	1026* ± 15	1031* ± 66	1017* ± 32	1001* ± 26	1013* ± 8
Citrate	1551 ± 65	1505 ± 21	1525 ± 54	1531 ± 23	1572 ± 21	1464 ± 27	1451 ± 50	1457 ± 31	1539 ± 62	1460 ± 55	1506 ± 20	1476 ± 36
Sulphate	116 ± 1	112 ± 7	110 ± 6	110 ± 12	109[c] ± 2	111 ± 4	104 ± 8	113 ± 8	107 ± 8	118 ± 11	110 ± 4	120 ± 15
Amount [mM]												
Sodium	16.6 ± 0.6	15.6 ± 0.2	16.3 ± 0.7	15.7 ± 0.4	16.9 ± 0.2	16.0 ± 0.2	15.8 ± 0.9	15.7 ± 0.04	15.8 ± 0.7	16.1 ± 9	16.7 ± 0.4	16.0 ± 0.2
Potassium	35.6 ± 1.1	37.0 ± 0.2	36.0 ± 1.2	37.5 ± 0.7	36.0 ± 1.9	37.7 ± 0.2	34.3 ± 2.4	37.1 ± 0.2	35.9 ± 1.8	38.2 ± 29	36.9 ± 0.9	37.4 ± 0.2
Magnesium	3.1 ± 0.2	3.5 ± 0.1	3.0 ± 0.1	3.5 ± 0.2	3.0 ± 0.04	3.7 ± 0.04	2.9 ± 0.1	3.6 ± 0.04	2.8 ± 0.1	3.7 ± 3	3.0 ± 0.04	3.4 ± 0.04
Calcium	8.6 ± 0.6	8.7 ± 0.2	8.6 ± 0.2	8.6 ± 0.4	8.1 ± 0.6	8.3* ± 0.1	7.0* ± 0.4	7.4* ± 0.2	6.6* ± 0.5	7.2* ± 6	6.8* ± 0.3	5.8* ± 0.1
Chloride	28.5 ± 0.2	26.1 ± 0.3	27.5 ± 0.5	26.3 ± 0.4	28.3 ± 0.3	26.3 ± 0.5	26.1 ± 1.6	25.9 ± 0.1	27.5 ± 1.6	26.5 ± 0.4	28.1 ± 0.5	26.1 ± 0.2
Phosphate	12.0 ± 0.1	12.0 ± 0.1	11.7 ± 0.6	11.8 ± 0.3	11.0* ± 0.4	11.6 ± 0.3	10.4 ± 0.8	10.6* ± 0.2	10.6* ± 0.7	10.5* ± 0.3	10.3* ± 0.3	10.4* ± 0.1
Citrate	8.1 ± 0.3	7.8 ± 0.1	7.9 ± 0.3	8.0 ± 0.1	8.2 ± 0.1	7.6 ± 0.1	7.6 ± 0.3	7.6 ± 0.2	8.0 ± 0.3	7.6 ± 0.3	7.8 ± 0.1	7.7 ± 0.2
Sulphate	1.2 ± 0.01	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1[c] ± 0.02	1.2 ± 0.04	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.1 ± 0.04	1.2 ± 0.2

[a]n = 3 for all filtration temperatures except 50 °C where n = 5; an asterisk indicates significantly (α = 0.05) lower amounts compared to means of UF permeate at 10 °C.

Tab. 6–3: Physical properties, dry matter and lactose content of ultrafiltration permeate obtained at different filtration temperatures.[a]

Analysis	Filtration temperature					
	10 °C	20 °C	30 °C	40 °C	50 °C	55 °C
$pH_{20\,°C}$ [-]	6.79	6.77	6.72	6.66	6.64	6.54
Dry matter [%]	5.62	5.61	5.61	5.60	5.61	5.65
Lactose [g L^{-1}]	49.4	49.8	49.6	49.8	49.5	49.3
Conductivity [mS cm^{-1}]	5.42	5.42	5.39	5.37	5.33	5.29
Freezing point [°C]	-0.4878	-0.4856	-0.4862	-0.4841	-0.4851	-0.4821

[a]n = 2.

Physical parameters that correlate with mineral composition and the amount of minerals present like conductivity and freezing point were found to decrease and increase with increasing filtration temperature, respectively. In terms of dry matter and lactose content, no differences could be observed. The transition of calcium and phosphate into the crystalline phase is accompanied by a decrease in pH of the solution giving a new equilibrium state in terms of calcium-to-phosphate ratio and pH in the serum phase.

Determination of total cations and anions in skim milk

Appropriate sample preparation for ion chromatography is also suitable for the determination of total cations and anions including citrate and lactate in skim milk. Thereby, a comparison of the ratio of soluble minerals in UF permeate to colloidally bound minerals can be performed. Tab. 6–4 shows the results of the analysis of the perchloric acid filtrates by ion chromatography. Perchloric acid precipitation and subsequent analysis showed a good reproducibility and comparability as the composition of bulk skim milk was assumed to be fairly constant over four weeks of sampling. Milk samples 1 and 2 were used for the filtration experiments. Sample 3 was included to show quantitative variation in individual milks in different batches of bulk skim milk in comparison to analytical variation.

Tab. 6–4:Total ionic composition of different milk samples determined by precipitation of proteins and solubilisation of colloidal salts using perchloric acid.[a]

Analyte	Amount		
	Sample 1	Sample 2	Sample 3
Sodium	405 ± 14	421 ± 3	416 ± 4
Potassium	1598 ± 7	1587 ± 22	1628 ± 11
Magnesium	166 ± 7	162 ± 2	160 ± 4
Calcium	1383 ± 42	1305 ± 12	1357 ± 21
Chloride	926 ± 12	953 ± 38	957 ± 43
Phosphate	1830 ± 98	1803 ± 38	1971 ± 38
Citrate	1622 ± 49	1558 ± 38	1620 ± 36
Sulphate	114 ± 5	113 ± 8	119 ± 6
Ca/PO_4 ratio	1.85	1.77	1.69

[a]Values are in mg L^{-1}; n = 3.

Protein content of milk samples 1, sample 2, and sample 3 was 3.75, 3.75 and 3.81, respectively. At 10 °C, about 27% of total calcium and 65% of total phosphate were present in the ultrafiltration permeates, whereas at 50 °C 21% of total calcium and 56% of total phosphate were present

in the serum. This means that at lower temperatures, the calcium-to-phosphate ratio tends more towards unity (0.72 at 10 °C) compared to higher temperatures (0.67 at 50 °C). During precipitation, calcium in milk serum becomes stoichiometrically the limiting factor for calcium phosphate precipitation. Magnesium was present to about 50% of total magnesium in the milk serum. Other soluble ions like sodium, potassium and chloride, citrate and sulphate were mainly present in the serum phase (> 86% of total ionic species) as also reported by Gaucheron (2005).

Theoretical ionic composition and stability of existing and proposed SMUF solutions

In our opinion, the SMUF solution developed by Jenness and Koops (1962) should be regarded as a starting point rather than a milk buffer solution that needs to be improved. The aim of this study was rather to evaluate the possible working range of the existing SMUF solutions in terms of pH and temperature by the determination of calcium activity measurements, to extend the range of SMUF solutions available, and to predict the onset of precipitation depending on the calcium activity in the soluble region when SMUF is compared to skim milk containing caseins. Tab. 6–5 shows a comparison of the two proposed SMUF solutions of Jenness and Koops (1962)a so-called *dry blend mixture* and *wet mixture* and SMUF solutions that were derived from the corresponding ionic composition of UF permeates obtained in this study.

Tab. 6–5 Calculated amounts of individual ionic species of different SMUF solutions; comparison with the SMUF solutions of Jenness and Koops (1962).[a]

Analyte	SMUF-JK *dry-blend mixture*		SMUF-JK *wet mixture*		SMUF 10 °C		SMUF 50 °C	
	[mg L^{-1}]	[mM]	[mg L^{-1}]	[mM]	[mg L^{-1}]	[mM]	[mg L^{-1}]	[mM]
Sodium	420	18.3	420	18.3	347	15.1	347	15.1
Potassium	1357	34.7	1540	39.4	1272	34.5	1376	35.2
Magnesium	78	3.2	78	3.2	71	2.9	71	2.9
Calcium	360	9.0	359	9.0	336	8.4	254	6.34
Carbonate	130	2.2	130	2.2	-	-	-	-
Chloride	1149	32.4	1150	32.4	1029	29.0	995	28.1
Phosphate	1126[b]	11.6	1104[b]	11.6	1143	11.8	1009	10.45
Citrate	1603	8.4	1851	9.6	1603	8.4	1509	7.9
Sulphate	99	1.0	99	1.0	110	1.15	110	1.15
Ca/PO_4 ratio		0.78		0.78		0.71		0.61

[a]Data for SMUF-JK dry-blend mixture calculated from the recipe of Jenness and Koops (1962); data for SMUF-JK wet mixture taken from Jenness and Koops (1962).
[b]Calculated as phosphate from phosphorus.

Results were calculated based in the amounts of individual salts added. SMUF 10°C closely resembles the *dry blend mixture* suggested by Jenness and Koops (1962) except for carbonate and final pH adjusted to 6.75 as observed in UF permeate. Of course, a lower pH will reduce the risk of spontaneous precipitation over time which should

be taken into account. The *wet mixture* was found to be more stable towards heat- and pH-induced precipitation. This might be attributed to the significantly higher amount of citrate present in solution which might result in a lower calcium activity and a higher solubility of calcium shown later in this study so that we have to state that these two solutions were not equal in composition as suggested by Jenness and Koops (1962).

The recipe for SMUF 10 °C and SMUF 50 °C was as shown in Tab. 6–1. SMUF 50 °C is suggested for working temperatures of 50 °C that might be used for diafiltration of milk for removal of lactose and/or whey proteins, rapid dialysis or particle sizing at that temperature. It contains lower amounts of calcium and phosphate and has a lower natural pH of about 6.65 but can also be used up to a pH of 7.2 at 25 °C without precipitation as shown later. It might also be used to dissolve or dilute milk protein concentrates that were obtained by micro- or ultrafiltration at about 50 °C. SMUF solutions for other temperatures can be found in the appendix section. Prolonged storage of SMUF solutions up to 3 hours at the corresponding temperature resulted in no observable precipitation.

Ionic composition and physical properties of existing and proposed SMUF solutions

To close the circle and to validate the preparation method and the mineral determination by the developed ion chromatography method, we also investigated the amounts of minerals present in SMUF solutions by ion chromatography. The resulting dataset is shown in Tab. 6–6 as means including standard deviations. All constituents were present in the amount expected except for sodium which was present to a lesser extent than calculated in the *wet mixture* and *dry blend mixture*. However, this resulted in amounts closer to UF permeate. Selected physical properties of the suggested SMUF solutions in comparison to the SMUF solutions suggested by Jenness and Koops (1962) are shown in Tab. 6–7.

Tab. 6–6 Ionic composition of different SMUF solutions as determined by ion chromatography.

Analyte[a]	SMUF-JK *dry blend mixture*	SMUF-JK *wet mixture*	SMUF 10 °C	SMUF 50 °C
Sodium	388 ± 5	367 ± 2	335 ± 6	350 ± 8
Potassium	1463 ± 3	1426 ± 1	1331 ± 8	1323 ± 14
Magnesium	90 ± 2	108 ± 3	87 ± 2	82 ± 5
Calcium	367 ± 8	345 ± 6	343 ± 17	279 ± 6
Carbonate	-[c]	-[c]	-	-
Chloride	1226 ± 10	1126 ± 3	991 ± 10	1021 ± 6
Phosphate	1165 ± 9	1121 ± 8	1159 ± 37	1010 ± 40
Citrate	1686 ± 35	1833 ± 85	1592 ± 64	1500 ± 27
Sulphate	130 ± 6	116 ± 9	115 ± 6	115 ± 3
Ca/PO_4 ratio	0.76	0.75	0.72	0.67

[a]Values in mg L^{-1}; n = 3 for all values except SMUF 50 °C where n = 5; a dash indicates not detected.

Freezing point, conductivity, and pH were reduced by addition of lactose to SMUF solutions. The amount of lactose added largely affected the freezing point so that we have to conclude that the freezing point is not a good means to estimate ionic strength or even ionic composition. This can also be concluded when these results are compared to the data obtained by Jenness and Koops (1962). Freezing points of SMUF solutions after lactose addition were still closer to zero than UF permeates in all cases. This indicates that low molecular weight NPN components will also cause additional freezing point depression. Effects on pH might also be caused by impurities in the lactose.

Tab. 6–7: Physical properties of different SMUF solutions with and without addition of lactose.[a]

Analysis[a]	SMUF-JK *dry blend mixture*		SMUF-JK *wet mixture*		SMUF 10 °C		SMUF 50 °C	
	– lactose	+ lactose[a]	– lactose	+ lactose[a]	– lactose	+ lactose[a]	– lactose	+ lactose[a]
$pH_{20\,°C}$ [-]	6.66	6.62	6.65	6.64	6.82	6.79	6.65	6.59
Dry matter [%]	0.48	5.37	0.46	5.35	0.50	5.27	0.44	5.24
Conductivity [mS cm^{-1}]	6.29	5.54	6.14	5.62	5.47	4.83	5.39	4.79
Freezing point [°C]	-0.2011	-0.4544	-0.1950	-0.4565	-0.1729	-0.4357	-0.1667	-0.4311

[a]Lactose concentration of 49.5 g L^{-1}.

Calcium activity in skim milk, UF permeate, and SMUF solutions

In addition to compositional aspects of SMUF solutions and physical properties, calcium activity was determined depending on pH and type of UF permeate and SMUF to follow the state of calcium ions and the saturation level of calcium phosphate in SMUF at room temperature over pH.

Calcium activity in milk and especially concentrated milk systems has received considerable attention in research in the recent years due to the effect of calcium on milk proteins (Lewis 2011). Especially milk systems that had undergone modifications by calcium fortification or by diafiltration with different water qualities were analysed for calcium activity in relation to protein stability. Calcium activity is considered to have a major impact on casein micelle integrity and stability. Fig. 6-2a shows a comparison in calcium activity over pH of UF permeates obtained at 10 °C and 50 °C with existing SMUF solutions of Jenness and Koops (1962). In Fig. 6-2, UF permeates are also compared with the suggested SMUF solutions without (Fig. 6-2b) and with (Fig. 6-2c) lactose addition. Fig. 6-2d shows the effect of soluble calcium addition as 10% calcium chloride solution on calcium activity. It can be seen in Fig. 6-2a that the *wet* and *dry mixture* of Jenness and Koops (1962) were different from each other in terms of their calcium activity probably due to the differences in citrate concentration. The *dry blend mixture* showed a calcium activity comparable to UF permeate obtained at 10 °C and a rapid decrease due to precipitation of calcium phosphate at pH > 6.8 which became visible due to turbidity of the solutions. The *wet*

mixture showed a lower calcium activity and a higher pH at which calcium phosphate starts to crystallize indicated by a sharp decrease in calcium activity.

Calcium activity is likely to be affected due to the Ca/PO_4 stoichiometric ratio of 1.5 of the precipitated material which is different from the ratio present in the liquid phase (Tab. 6–6). Calcium activity of SMUF 10 °C and SMUF 50 °C solutions in Fig. 6-2b differed also slightly from the results of UF permeate. SMUF 10 °C showed lower calcium activity than UF permeate obtained at 10 °C. This difference could be explained by one major difference in composition of UF permeate and SMUF solutions which is the presence of lactose. Therefore, lactose was also added to SMUF 10 °C and SMUF 50 °C and calcium activity was determined depending on pH.

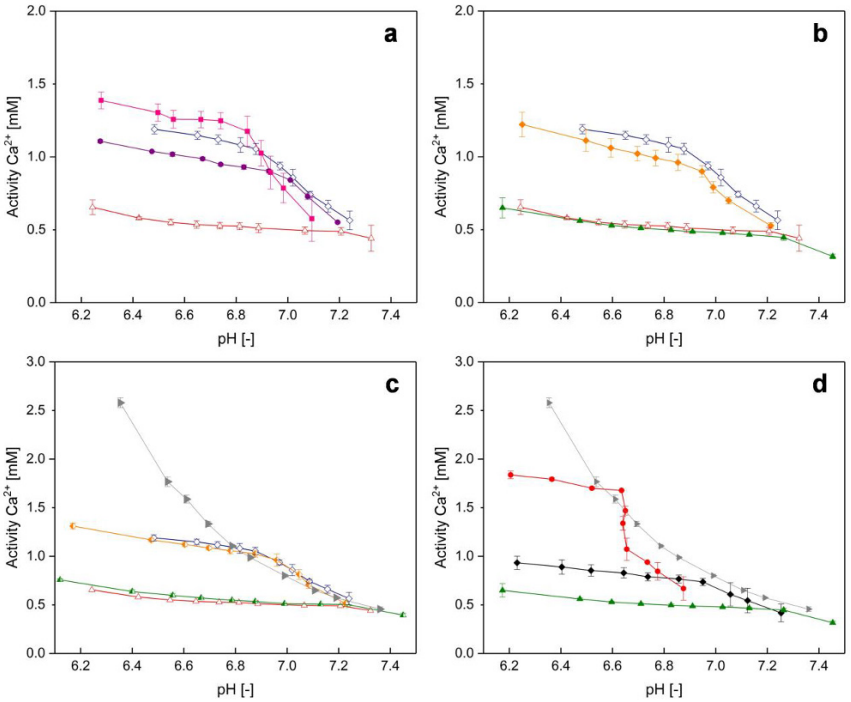

Fig. 6-2: Ca^{2+}-activity over pH for SMUF solutions of Jenness and Koops (1962) (a) as wet blend mixture (—●—) and dry blend mixture (—■—) in comparison to UF permeate obtained at 10 °C (—◇—) and 50 °C (—△—). Calcium activity of SMUF 10 °C (—◆—) and SMUF 50 °C (—▲—) solutions without (b) and with lactose addition (c) are shown in comparison to UF permeate 10 °C and 50 °C. Ca^{2+} activity (d) of SMUF 50 °C to which 1 mM Ca2+ (—◆—) and 3 mM Ca^{2+} (—●—) were added in comparison to SMUF 50 °C without additional calcium (—▲—). Ca^{2+} activity of pasteurised skim milk (—▶—) is shown for comparison.

Addition of lactose to SMUF 10 °C increased the calcium activity so that it became equal to that of UF permeate at 10 °C as shown in Fig. 6-2c. Thereby, differences in

calcium activity of SMUF without lactose could be explained and the suggested SMUF solutions came very close to the calcium activity of UF permeate obtained at 10 and 50 °C. The effect of lactose on calcium activity might be due to the interaction of lactose with citrate and phosphate ions or excluded volume effects as suggested by Gao et al. (2010c). Thereby, a comparably small proportion of calcium becomes detectable.

This effect was found to be comparably small when we consider the effect of pH on calcium activity. Calcium addition to SMUF 50 °C as shown in Fig. 6-2d resulted in a higher calcium activity in the solubility region. A comparison of SMUF solutions and UF permeate to the calcium activity in pasteurised skim milk Fig. 6-2c and d shows that at higher pH than the 'natural' pH of 6.75 of UF permeates obtained at 10 °C at which precipitation is induced, calcium activity of skim milk is very similar to UF permeates. At lower pH than the natural pH of skim milk ($pH_{20\,°C}$ 6.72-6.77), calcium and phosphate are released from the casein micelles and calcium activity therefore increases until saturation of the serum phase is accomplished. A slight increase of calcium activity in the undersaturated region in UF permeates was observed which might result from the protonation of citrate and phosphate and a subsequent release of complexed calcium.

The natural pH of UF permeate obtained at 50 °C is likely be the result of a shift of the region of supersaturation at higher temperatures than 25 °C to a lower pH which then results in saturation at a higher pH at lower temperature. This indicates that the limit of saturation of UF permeates derived from skim milk can be determined by calcium activity measurements. The point of saturation of a SMUF solution with respect to calcium phosphate at a certain temperature will be indicated by a sharp decrease in calcium activity over pH. For scientific purposes, a SMUF solution that is close to the solubility limit at a certain temperature should be used when equilibrium with casein micelles is to be obtained as the saturation criterion in UF permeates is only valid at the temperature and pH conditions of the filtration process.

6.4 Conclusions

Mineral equilibria in milk can be approximatively simulated by a synthetic milk salt solution. The critical pH of calcium phosphate precipitation and the equilibrium pH of SMUF with casein micelles at a certain temperature, i.e. skim milk, at room temperature were found to be predictable by calcium activity measurements. Analysis of permeates of milk at a certain pH and temperature by ion chromatography complemented by calcium activity measurements offers the possibility to approximate the equilibrium conditions by a synthetic milk salt solution. Therefore, SMUF solutions should be considered as dynamic in composition depending on the case of application and the scope of the study to be undertaken for compositional changes in the milieu of milk proteins by any preparative technique.

Regions of supersaturation depending on composition of the SMUF solution have to be investigated prior to setup of experiments and could be estimated by calcium activity measurements in comparison to skim milk. For convenience, a method of preparation was suggested that is easy to handle and also highly flexible in the variation of individual components which was not scope and performed in detail in this study. Nonetheless, properly defined SMUF solutions with adjusted calcium activity could be used to work out a correlation of calcium activity as a function of pH and temperature with casein micelle stability. The ideal SMUF solution will have amounts of calcium and phosphate, a pH at a certain temperature, and a citrate level that results in saturation at that temperature. Any SMUF solution is therefore an approximation to the state of UF permeate. Finally, simplified SMUF solutions could also be applied as diafiltration media in industry and result in heat stable and functional casein and whey protein fractions.

Appendix

Preparation of SMUF solutions for other temperatures

Weight [g L^{-1}]			Amount [mM; SMUF 30 °C/SMUF 40 °C]							
SMUF 30 °C	SMUF 40 °C	Component	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	PO_4^{3-}	Citrate	SO_4^{2-}
0.839	0.833	KH_2PO_4		6.56/6.1				6.6/6.1		
0.813	0.767	K_2HPO_4		9.34/8.8				4.7/4.4		
0.7		$K_3C_6H_5O_7 \cdot H_2O$		4.9					2.1	
1.2		$Na_3C_6H_5O_7 \cdot 2\ H_2O$	12.2						4.0	
0.6		$Mg_3(C_6H_5O_7)_2 \cdot 9\ H_2O$				2.9			1.9	
0.057		$C_6H_8O_7 \cdot H_2O$							0.3	
0.733	0.867	KCl		9.8/11.6			9.8/11.6			
0.167		NaCl	2.9				2.9			
0.2		K_2SO_4		2.3						1.1
1.2	1.05	$CaCl_2 \cdot 2\ H_2O$			8.2/7.12		16.3/14.2			
0.07	0.06	KOH		1.3/1.1						
Sum			15.1	35.0/34.9	8.2/7.1	2.9	27.2/28.7	11.2/10.5	8.4	1.1

Summary and contribution of the doctoral candidate

Simulated milk ultrafiltrate (SMUF) is a synthetic salt solution that simulates the ionic composition in the serum phase of skim milk which can be obtained as ultrafiltration permeate. SMUF was used in research for multiple purposes to investigate the effect of single milk components on, e.g., heat stability of milk proteins, or for dilution of casein micelles for physical analysis. As casein micelles are known to be sensitive towards changes in ionic composition, especially soluble calcium, temperature,

and pH in their surrounding medium, structural changes of casein micelles can be investigated by modification of this SMUF solution. Due to the dynamic equilibrium of the serum phase with casein micelles, ultrafiltration permeates were known to change with respect to calcium and phosphate content. However, an existing SMUF solution with fixed composition, based on ultrafiltration (UF) permeate obtained at room temperature, was found to show spontaneous crystallisation of calcium phosphate and therefore to interfere with experiments, especially when used at higher temperature or adjusted to higher pH.

Therefore, the aim of this study was to develop SMUF solutions according to the composition of UF permeates obtained at different temperatures and analysed by ion chromatography for the amount of individual cations and anions as well as other physico-chemical characteristics. Supersaturation of different UF permeates and SMUF solutions with respect to calcium phosphate depending on pH was investigated by calcium activity measurements and compared to skim milk.

The results of this study showed that SMUF solutions, with and without added lactose, could be prepared that simulate UF permeate at different temperatures. The corresponding amounts of individual ions were found to be similar. In addition, calcium activity which indicates the amount of soluble non-complexed calcium in the serum and the onset of calcium phosphate precipitation was similar for SMUF solutions as compared to their corresponding UF permeate. Calcium activity was therefore found to be a useful tool to estimate the onset of calcium phosphate precipitation depending on soluble calcium, pH, and temperature. The saturation point with respect to pH of UF permeates and SMUF at a certain temperature can be estimated by comparison with the calcium activity in skim milk depending on pH.

The major contributions of the doctoral candidate were the development of the SMUF solutions based on literature and own analytical data. Scientific assistance for the development of the ion chromatography method and validation after critically reviewing the existing literature was given by the doctoral candidate. The calcium selective electrode measurement procedure was established by the doctoral candidate. Next to this, the doctoral candidate carried out data analysis, interpreted datasets, acquired and plotted data for this work. The manuscript was essentially written by the doctoral candidate.

7 Modelling of the heat stability of concentrated milk[10]

Abstract

Heat stability is known to be limited in concentrated skim milk (CSM) and was often found to be in conflict with inactivation of bacterial spores by thermal treatment. Therefore, we investigated heat-induced coagulation of non-preheated and preheated CSM depending on total solids and heating temperature over time. The Weibullian model to describe the course of coagulation of casein micelles in concentrated skim milk of different total solids and at different heating temperatures made it possible to calculate a maximum heating temperature-time-total solids relationship based on a certain maximum allowance of heat-induced damage on casein micelles.

10 Original publication: Dumpler J, Peraus F, Depping V, Stefánsdóttir B, Grunow M, Kulozik U (2017) Modelling of heat stability and heat-induced aggregation of casein micelles in concentrated skim milk using a Weibullian model. **Submitted** to Int J Dairy Technol. Adapted original manuscript. Adaptions of the manuscript refer to title, enumeration type, citation spelling, notation of units, and format.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_7

7.1 Introduction

Concentrated skim milk (CSM) in the range of 20 up to 35% total solids has recently been proposed as a substitute for skim milk powder wherever the latter is reconstituted, for example when producing yoghurts, ice cream, filled bakery products, or finished meals. From an environmental standpoint, switching to CSM is advantageous, even if CSM is trucked up to 1000 kilometres (Depping et al. 2017). Among other factors, the shelf life is determined by the microbial inactivation of bacterial spores from applied thermal treatments.

A critical variable limiting possible heat treatments of CSM with various total solids contents is its corresponding heat stability. Heat stability of milk and concentrated milk is defined as the time required inducing coagulation of the milk proteins at a certain temperature, usually above 100 °C. This induction of coagulation is mostly detected visually, i.e. this method is called the 'subjective' method, and the corresponding time is recorded as heat coagulation time to simulate in-container sterilisation (Davies and White 1966). Attempts to quantitatively follow heat-induced coagulation as an ongoing process by analytical techniques to investigate the onset, course, and extent of protein aggregation have been performed to a much lesser extent than the subjective test method (Whitney et al. 1952; White and Davies 1966; White and Sweetsur 1977; Nieuwenhuijse et al. 1991). Nevertheless, the point of interest was in most cases the onset of coagulation, i.e. the timespan separating the stability zone from the region of 'uncontrolled' coagulation.

Retorting of milk products is still of practical relevance, but is becoming increasingly replaced by continuous heating systems (Muir 1984; Hinrichs et al. 1998; Hinrichs 2000). Heat-induced coagulation of CSM in continuous heating systems cannot be predicted by the subjective heat stability test, since heat-induced coagulation was also found to appear during continuous UHT heat treatments at short holdiung times and high temperature (Hinrichs et al. 1998; Smith and Malmgren 1999; Dumpler and Kulozik 2016). Therefore, kinetic data could significantly improve the predictability of heat-induced coagulation independent of the heating system used and the temperature-time profile of the equipment. The choice of temperature-time combinations including heat-induced coagulation as an undesired physical destabilisation of milk proteins in concentrated milk systems could not be taken into consideration for the optimisation of thermal processes. Therefore, heat stability has most often been tested empirically by single industrial or pilot scale trials (Smith and Malmgren 1999; Dumpler and Kulozik 2016). Efforts made by Dumpler and Kulozik (2016) to estimate heat stability on pilot scale using direct steam injection to perform isothermal heat treatments were limited to short and fixed holding times. This limited the processing window and did not allow for the prediction of non-isothermal heat treatments and holding times > 20 s. Therefore, we considered approach to following coagulation over time to derive kinetic parameters as worthwhile.

White and Sweetsur (1977) first modelled heat-induced coagulation by using a formal second order reaction kinetics approach. They investigated heat-induced co-

agulation of unconcentrated milk in the temperature range of 110-140 °C. They found that the activation energy for heat coagulation of unconcentrated milk was ~125 kJ mol^{-1}. Nevertheless, this result was valid, if only the coagulation process was modelled. The preceding *lag*-phase and the subsequent stationary phase without further coagulation were not included in the model. In addition, a non-normal distribution of residuals was observed in some cases which indicate first order kinetics to be unsuitable for modelling this reaction. This may be due to multiple reactions taking place until visual coagulation occurs and proceeds. These multiple effects render it virtually impossible to quantify single effects and describe heat-induced coagulation as a sum of fostering and adverse reactions taking place eventually leading to coagulation. A Weibullian model of the shape $f(x) = a - (a - b) \cdot e^{-(k \cdot t)^c}$ that was recently used to describe the inactivation of microbial populations could also be used to describe the complex phenomenon of aggregation of a population of casein micelles in milk.

This Weibullian model is able to account for all three stages of the coagulation process, the *lag*-phase, the proceeding coagulation, and the plateau phase when a lower asymptote b, a upper asymptote a, and a shape parameter c are included. The shape parameter c together with the rate constant is able to model the length of the *lag*-phase without significant coagulation. Weibullian models were used to increase the precision of modelling microbial inactivation where shoulder formation was observed, i.e. slow inactivation at the beginning and continuous thermal damage increasing the rate of inactivation over time. Log-linear models were found to be unsuitable to represent this complex behaviour whereas the Weibullian model is basically a statistical model of the distribution of inactivation times (van Boekel 2002).

Hence, the aim of this study was to model the extent of heat-induced destabilization of casein micelles and subsequent coagulation of CSM with various total solids contents for both onset and course of coagulation over time at different temperatures. Kinetic parameters should be estimated and used to inter- and extrapolate data for predicting the coagulation of CSM at higher total solids, higher heating temperatures, and shorter holding times.

7.2 Materials and methods

Preheat treatment and concentration of skim milk by reverse osmosis (RO)

Pasteurised (74 °C/28 s) bulk skim milk was obtained from a local dairy factory (Molkerei Weihenstephan, Freising, Germany). Average main composition was as described by Dumpler and Kulozik (2015). Preheated and non-preheated skim milk was concentrated by reverse osmosis (RO) to achieve different a total solids content of the CSM in the range from 12% to 33% was as describe by Dumpler and Kulozik (2016). A small amount of RO permeate was added if necessary to adjust the final total solids content determined using a microwave dryer (CEM Smart Turbo 5, CEM, Kamp-Lintfort, Germany). The initial pH of CSM before heating was as described by Dumpler and Kulozik (2016).

Heat treatment, dilution, and centrifugation of the samples

Heat treatment of the concentrated skim milk was performed similarly as described by Bulca et al. (2016). In short, stainless steel tubes with an inner diameter of 10 mm, a wall thickness of 0.5 mm, and a length of 195 mm resulting in a volume of 15 mL were filled with CSM of different total solids and heat treated at temperatures in the range from 103 °C to 131 °C by saturated steam for up to 5,000 s. The holding times were chosen in regular intervals depending on the expected heat stability of the CSM. Heating and cooling from room temperature took approximately 40-60 s each and was therefore negligible compared to the holding times used. Instant heating is necessary to derive kinetic parameters from the resulting isothermal heat treatments. Fouling of the tubes was not observed. However, the viscosity changes of the CSM after heat treatment was remarkable in some cases. Especially when samples of high total solids content were heat treated, advanced coagulation had led to far reaching structural changes. Therefore, heat treated samples were diluted approximately 1:3.5 with distilled water after heat treatment prior to centrifugation as coagulated protein was not completely sedimentable at high viscosity after heat treatment. Complete sedimentation of aggregated protein was monitored by particle size measurements. After dilution, a complete sedimentation of protein aggregates was possible. Diluted samples in 50 mL Falcon tubes were centrifuged in a bench-top laboratory centrifuge at 4,000*x*g/10 min. Supernatants were removed and analysed for non-sedimentable protein as described by Dumpler and Kulozik (2016). Sedimentable protein is defined by the relative amount of protein that is separated under these conditions. The relative amount of non-sedimentable protein was calculated taking into account the dilution of each sample compared to the sample that was heated and cooled immediately after reaching the desired temperature. The unheated sample was analysed but not used as a reference for calculation of the relative amount of sedimentable protein as CSM with high total solids showed coagulation of about 10% of the protein even after heating and immediate cooling. This is the reason why higher CSM total solids and higher temperatures could not be investigated using the heating system described.

Supernatants were analysed for soluble caseins by RP-HPLC and were also further fractionated by ultracentrifugation at 48,800*x*g/26 min. Centrifugal and ultracentrifugal supernatants were analysed for caseins as described by Dumpler et al. (2017c). Heat treatments of two individual samples at each temperature-time-totals solids combination were performed at least in duplicate and each sample was analysed (n = 4). Experiments for concentrates from preheat treated milk were single determinations.

RP-HPLC analysis of samples

Centrifugal and ultracentrifugal supernatants were analysed for soluble caseins after dilution in guanidine buffer for complete solubilisation of proteins as described by Dumpler et al. (2017c) with modification of the elution gradient. It was observed that whey protein peaks became blurred and 'disappeared' from the chromatograms after about 200 s of heating. We assume that this is due to glycosylation, covalent cross-

linking, Maillard-type reactions, and other chemical reactions taking place in the concentrated system at high temperatures. Therefore, the partition of whey proteins within the fractions could not be investigated. Hence, the gradient was increased so that residual whey proteins eluted in the washing step and the method was shortened to 29 min run time. In short, solvent A consisted of 0.1% trifluoroacetic acid (TFA) in 90% HPLC grade water and 10% acetonitrile, solvent B was 0.07% TFA in 10% HPLC grade water and 90% acetonitrile. The flow rate was 1.2 mL min^{-1}, the column temperature was kept at 40 °C, and detection was made at a wavelength of 226 nm. The gradient was as follows: linear from 27 to 32% B in 2 min, 32 to 45% B in 23 min, 38% B for 3 min, from 45 to 100% B in 1 min, then rinsing with 100% A for 2 min, and returning to 27% B within 1 min. As injection volume, 10 µL was chosen for centrifugal supernatants and 80 µL for ultracentrifugal supernatants. Standard deviations were calculated from two individual heat treated samples of two replicate trials (n = 4).

Data analysis and modelling of heat-induced aggregation using a Weibullian model

Data was plotted and statistical analysis was performed using OriginPro 2017G (OriginLab Corporation, Northampton, MA, USA). The Weibullian model for fitting the data was

$$f(x) = a - (a - b) \cdot e^{-(k \cdot t)^c} \tag{7.1}$$

where a is the lower asymptote, b is the upper asymptote of the model, k is the rate constant, and c is the shape parameter of the Weibullian model. The equation used in this study to model heat-induced aggregation of casein micelles in CSM can then be written as

$$\frac{C_t}{C_0} = \frac{C_\infty}{C_0} - \left(\frac{C_\infty}{C_0} - \frac{C_0}{C_0}\right) \cdot e^{-\left(\frac{t}{\alpha_{w,T,s}}\right)^{\beta_w}} \tag{7.2}$$

where C_t is the protein concentration at time t, C_0 is the initial protein concentration, C_∞ is the concentration of protein soluble in the stationary state when all proteins prone to coagulation have integrated into protein particles, $\alpha_{w,T,s}$ is the characteristic time of the Weibullian model that is dependent on both, heating temperature T and total solids content s of CSM, and β_w is the shape parameter. The parameter α_w would correspond to the D-value if formal first order kinetics would apply. In a first attempt to plot the normalized data, $\frac{C_\infty}{C_0}$, $\frac{C_0}{C_0}$, $\alpha_{w,T,s}$, and β_w were set as variables. In order to obtain values for $\alpha_{w,T,s}$, in a second approach β_w was set to a fixed value of 1.5 as averages from the first approach (as will be described later in section 7.3), so that the model consisted of three dependent variables to be determined.

The hazard function h(t) indication the failure rate of the Weibullian model is then

$$h(t) = \frac{\beta_w}{\alpha_w^{\beta}} \cdot t^{\beta_w - 1} \tag{7.3}$$

which increases over time when $\beta_w > 1$ indicating a continuous destabilisation of remaining casein micelles in CSM.

Discrete values were obtained from the fit for different temperatures at constant total solids content of 27% as well as for different total solids content at a heating temperature of 116 °C using eq. 7.2. Values of α_w over temperature and total solids were then analysed by regression and regression parameters were used to model the relationship between heating temperature, heat holding time and CSM total solids content at a fixed maximum level of sedimentable protein of 2% of total protein. Therefore, eq. 2 was rewritten as

$$\frac{C_t}{C_0} = \frac{C_\infty}{C_0} - \left(\frac{C_\infty}{C_0} - 1\right) \cdot e^{-\left(\frac{t_R}{\alpha_{w,T,s}}\right)^{1.5}} \tag{7.4}$$

where t_R is the 'reliable lifetime' in the Weibullian model that describes the heating time required to induce 2% of total protein to form sedimentable aggregates at a certain CSM total solids content and heating temperature (van Boekel 2009). When $\frac{C_\infty}{C_0}$ is known from the experiments, the reliable lifetime can be derived by solving equation (4) for t_R which results in eq. 7.5

$$\ln\left(\frac{\frac{C_t}{C_0} - \frac{C_\infty}{C_0}}{1 - \frac{C_\infty}{C_0}}\right) = -\left(\frac{t_R}{\alpha_{w,T,s}}\right)^{1.5} \tag{7.5}$$

and subsequently in eq. 7.6

$$t_R = \left(\ln\left(\frac{1 - \frac{C_\infty}{C_0}}{\frac{C_t}{C_0} - \frac{C_\infty}{C_0}}\right)\right)^{\frac{1}{1.5}} \cdot \alpha_{w,T,s} \tag{7.6}$$

when a level of 2% protein of total protein is set as a fixed criterion $\frac{C_t}{C_0} = 0.98$ and $\frac{C_\infty}{C_0}$ is set as 0.48 (for CSM ≥ 18% totals solids), then eq. 7.6 becomes

$$t_R = (\ln\ 1.04)^{\frac{1}{1.5}} \cdot \alpha_{w,T,s} = 0.1153 \cdot \alpha_{w,T,s} \tag{7.7}$$

The parameter $\alpha_{w,T,s}$ can be derived by linear regression of $\alpha_{w,T}$ and $\alpha_{w,s}$ over temperature and CSM total solids, respectively. Two reference points need to be defined as $\alpha_{w,T_{ref}}$ and $\alpha_{w,s_{ref}}$. These reference points were the values for $\alpha_{w,T}$ at 116 °C and $\alpha_{w,s}$ at 27% total solids derived from the regression of the variation in temperature and total solids content, respectively. These data points were approximately equal so that $\alpha_{w,T_{ref},s_{ref}}$ can be defined as equal to 300 s. Then the two factors f_T eq. 7.8 and f_s eq. 7.9 can be defined as

$$f_T = \frac{\alpha_{w,T}}{\alpha_{w,T_{ref}}} \tag{7.8}$$

and

$$f_s = \frac{\alpha_{w,s}}{\alpha_{w,s_{ref}}} \tag{7.9}$$

These were then used to calculate $\alpha_{w,T,s}$ as shown in eq. 7.10

$$\alpha_{w,T,s} = \alpha_{w,T_{ref},s_{ref}} \cdot f_T \cdot f_s \tag{7.10}$$

This parameter is then used to calculate the reliable lifetime of CSM until the onset of coagulation eq. 7.7.

The *z*-values for a decrease of the parameter $\alpha_{w,T}$ by 90% with increasing temperature by a certain difference were calculated from the slope of the regression of the Arrhenius diagrams. The *z*-values was determined by eq. 7.11

$$z = \frac{1}{\frac{1}{T_{ref}} - \frac{\Delta \log a_{w,T}}{\mu_{\alpha_{w,T}}}} - T_{ref} \tag{7.11}$$

where T_{ref} is the reference temperature of 116°C, i.e. 389.15 K, $\mu_{\alpha_{w,T}}$ is the slope of the regression line in the Arrhenius diagram, and $\Delta \log a_{w,T}$ is by definition equal to unity. In addition, the Q_{10} value was calculated as it is often used in older literature for comparison. It is linked to the *z*-value by eq. 7.12

$$Q_{10} = 10^{\frac{10}{z}} \tag{7.12}$$

The activation energy E_A of the reaction was calculated as

$$E_A = \frac{2.303 \cdot R \cdot T_{ref} \cdot T^*}{z} \tag{7.13}$$

where R is the universal gas constant and T^* is the reference temperature plus 10 K.

7.3 Results and discussion

Amount of sedimentable protein depending on total solids content and heating temperature

The investigation of heat stability of concentrated skim milk (CSM) is based on the observation that casein micelles will form discrete large sedimentable particles that can be quantitatively separated from the heat treated concentrate at 4,000*x*g/10 min after dilution. The amount of sediment formed indicates the degree of destabilisation of CSM due to heat treatment over time. Early studies on heat stability using such an objective test method have indicated that unconcentrated and concentrated milk can withstand a certain period during heating without any protein aggregation. This period is the followed by a sharp decrease in soluble protein over time and subsequently a stationary phase when all proteins prone to coagulation have aggregated. White

and Davies (1966) used this observation to justify the use of the subjective heat stability test due to the ease of a visual detection of coagulation as the change at the onset of coagulation is generally severe. This behaviour was especially observed for CSM in this study as shown in Fig. 7-1. Data was plotted on a semi-logarithmic scale and fitted by the Weibullian model. The relative amount of sedimentable protein over the logarithm of heating time is shown depending on heating temperature at a constant total solids (TS) content of 27% (Fig. 7-1a). Fig. 7-1b shows the course of the relative amount of sedimentable protein over time at a constant heating temperature of 116 °C for various TS of CSM.

It can be seen from the graphs that with an increasing heating temperature and increasing total solids content of the CSM, the time is shortened until coagulation sets in and the extent of coagulation in relative quantities is in the range of 50-60% at < 15% total solids. The Weibullian model (equation 2) fitted the data with correlation coefficients of $R^2 > 0.95$ in all cases and $R^2 > 0.98$ in most cases. The residuals were found to be normally distributed using a normal probability plot and a Kolmogorov-Smirnov test (Appendix). This fitting procedure was also performed for CSM from preheated skim milk and parameter estimates will be presented further down.

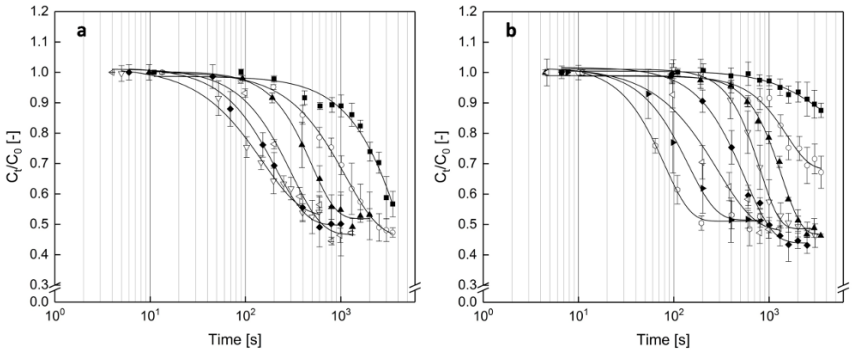

Fig. 7-1: Relative amount of sedimentable protein over the logarithmic heating time depending on heating temperature at 27% CSM total solids (a) and depending on total solids at 116 °C (b) fitted by the Weibullian model. Heating temperatures *(left)* were 104 (■), 108 (○), 112 (▲), 116 (◁), 120 (◆), and 124 °C (▽). Total solids content of the CSM (*right)* was 12 (■), 15 (○), 18 (▲), 21 (▽), 24 (◆), 27 (◁), 30 (▲), and 33% (○).

The Weibullian model is suitable to describe the sum of failure of individual entities over time under stress. Very often, initial stability towards stress results in a low failure rate at the beginning, an exponential increase in individual failures over time due to wear or continuous damage, and finally an asymptotic trend until all entities have failed. This non-linear model has also become popular for the heat inactivation of bacterial vegetative cells that show a distribution of resistance to thermal stress due to natural variation (van Boekel 2009). A transfer of this model to casein micelles in milk and concentrated milk under heat therefore seemed to be likely when we con-

sider that heat-induced aggregation is the final step in heat-induced coagulation. Coagulation of micelles is preceded by chemical and physical reactions that destabilise casein micelles. Destabilisation eventually leads to a condition where, after collision of the protein particles sufficient to overcome the electrostatic energy barrier, attractive forces (by hydrophobic or electrostatic interactions) become stronger than repulsive forces and lead to coagulum formation. This coagulum can form discrete particles or a gel structure depending on milieu conditions and shear (Nieuwenhuijse et al. 1992). Using the stainless steel tubes for static heating of CSM, shear forces were absent. Therefore, perikinetic aggregation dominates, which is aggregation based on Brownian motion.

Parameter estimates for protein aggregation using a Weibullian model

The modelling approach for the heat-induced aggregation based on first order kinetics taken by White and Sweetsur (1977) was only partly satisfactory as it was only capable to model the aggregation process after an induction period which is particularly relevant for heating processes designed to keep sample stability. Therefore, in this study, the regression of the data in Fig. 7-1 by a Weibullian model was performed. Four independent variables were used, i.e. discrete data needed to be defined for the upper and lower asymptote, the characteristic time α_w, and the shape parameter β_w. At 12 and 15 % total solids, the lower asymptote was not reached within the heating times investigated. Therefore, we tentatively used the lower asymptote predicted by the Weibullian model for further calculations as summarized in Fig. 7-2.

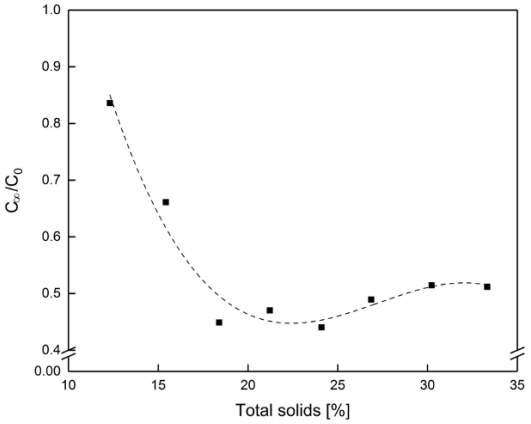

Fig. 7-2: Variation of the parameter *a* which represents the lower asymptote $\frac{C_\infty}{C_0}$ of the Weibullian model depending on total solids.

For all other CSM total solids, we used the mean of $\frac{C_\infty}{C_0} = 0.48$ as a lower asymptote of the Weibullian model. This is in the rage of literature reports of residual protein in

milk after complete coagulation. Whitney et al. (1952) have investigated the progress of coagulation of unconcentrated milk over time by removing coagulated protein by filtration techniques and subsequent determination of residual protein in the filtrate. They observed that about 50-60% of the protein coagulated upon prolonged heating. This test method was later refined by White and Davies (1966) using centrifugation of the samples after heat treatment for removal of the coagulum. White and Davies (1966) observed differences in the amount of coagulable protein between unconcentrated milk of individual cows whereby short coagulation times resulted in 'poor coagulation' and a maximum percentage of sedimentable protein of about 50% as observed by Whitney et al. (1952). The *lag*-time, i.e. heat stability, the extent of coagulation, and the progression of coagulation varied between individual cows. Nieuwenhuijse et al. (1991) found a relative amount of coagulable protein for non-preheated concentrated milk at 20% total solids independent of pH of about 50% which confirms the findings of this study.

The results for the shape parameter β_w for different heating temperatures at 27% CSM total solids and at 116 °C for different CSM total solids are shown in Fig. 7-3a and Fig. 7-3b. As the parameter β_w was very sensitive towards experimental error of single data points as observed by fitting data of single experiments, we decided to average the individual parameters $\beta_{w,T,s}$ of different heating temperatures and total solids content which resulted in $\beta_w \approx 1.5$.

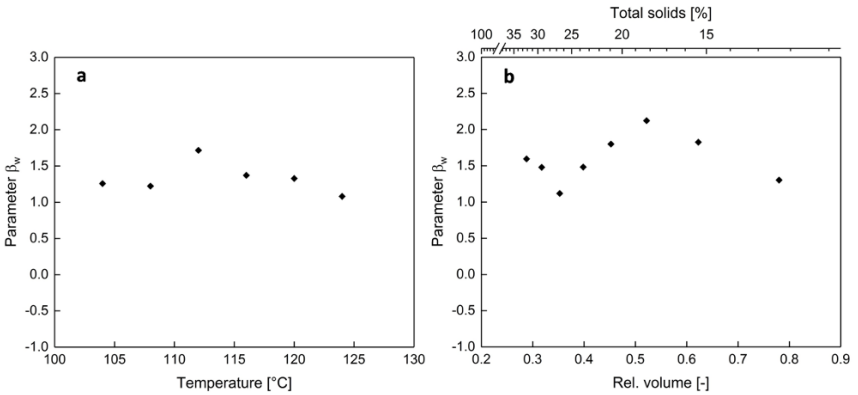

Fig. 7-3: Parameter β_w of the Weibullian model obtained by non-linear regression of sedimentable protein depending on heating temperature (a) and total solids content or the relative residual volume of the concentrate compared to unconcentrated skim milk (b).

Results of the lower and upper asymptote did not change on average when β_w was fixed. Results for α_w as characteristic time of the Weibullian model are shown in Fig. 7-4a. An Arrhenius plot over the reciprocal temperature is shown. Data with variable β_w and β_w fixed to 1.5 for non-preheated CSM of 27% total solids is shown. It can be seen from the comparison of the plot with fixed β_w and variable β_w that the effect of

a fixed β_w on α_w was comparably small. The graph shows that an Arrhenius dependency in terms of the characteristic time α_w can be assumed. Dotted lines show the 95% confidence intervals which indicate that the parameter α_w will be in 95% percent of replicated heat treatments within this interval. The interval has a certain width within the range investigated so that the characteristic time varies by a factor of ± 30% calculated from the mean at a certain temperature. Of course, α_w and β_w may vary considerably depending on milieu conditions like pH, ionic strength, ionic calcium, whey protein content, as well as technological treatments like homogenisation and preheating of the milk prior to concentration.

As one example, α_w for ultra-high temperature (UHT) preheated skim milk including 90 °C/80 s pre-holding and heating at 142 °C for 5 s prior to concentration to 27% CSM total solids (β_w =1.5) is also shown in Fig. 7-4a for comparison. It can be seen that preheating of milk results in an increase in heat stability of the concentrates by increasing the characteristic time α_w, especially at higher temperatures. This corresponds to an extension of the time required to induce noticeable coagulation and a reduced rate of aggregation when the shape parameter β_w is constant.

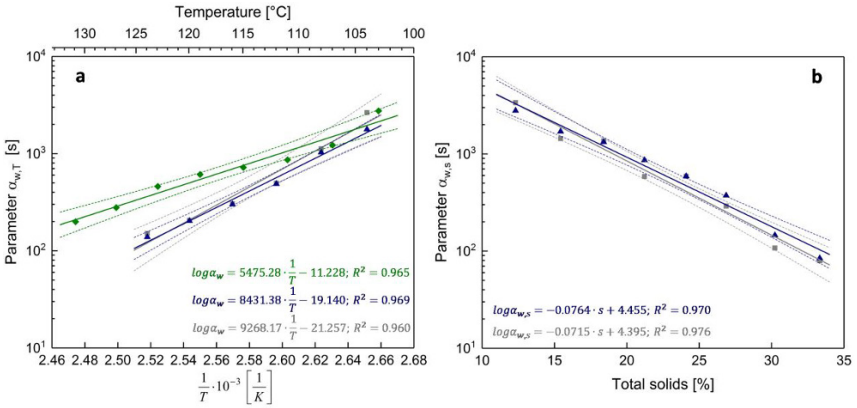

Fig. 7-4: Arrhenius-plot of the parameter α_w of the Weibullian model obtained by non-linear regression of sedimentable protein depending on heating temperature (a) and parameter $\alpha_{w,s}$ depending on total solids content (b) of CSM. Linear regression of parameter α_w over the inverse absolute temperature and total solids was performed including the 95% confidence interval *(dashed lines)* of values obtained for α_w at a variable β_w (■) and $\beta_w = 1.5$ (▲) for non-preheated CSM as well as preheated milk with $\beta_w = 1.5$ (◆) over temperature.

The characteristic time α_w is considerably shortened as total solids content of CSM is increasing as shown in Fig. 7-4b. Again, a linearization of the data over CSM total solids was possible for both cases, with variable β_w or $\beta_w = 1.5$. The findings on the close relationship between heating temperature, time, and total solids are generally in agreement with the findings of our earlier studies using the subjective lab scale heating system and using direct steam injection for heating (Dumpler and Kulozik

2015, 2016). The linear extrapolation of α_w over total solids (s) to zero percent total solids in Fig. 7-4 b is, of course, only meaningful when we consider $\lim_{s\to 0} \frac{C_\infty}{C_0} = 1$ as shown in Fig. 7-2.

The regression parameters of the Arrhenius-plot of α_w and the plot over total solids are indicated in the panels of Fig. 7-4 and kinetic parameters derived from the linear regression are indicated in Tab. 7–1 for the Arrhenius plot and in Tab. 7–2 for different CSM total solids. A z-value of 17.1 and 18.8 K, which corresponds to an activation energy of 177.5 and 161.4 kJ mol^{-1}, respectively, were calculated for the temperature dependency of the reaction with variable β_w and $\beta_w = 1.5$. Kessler (1975) and Walstra et al. (1984) included a coagulation area of casein micelles in milk into the temperature-time diagram for unconcentrated milk. According to this line, the maximum heat coagulation time of unconcentrated milk is about 10 min at 135 °C and the *z*-value is approximately 15 K.

Tab. 7–1: Parameters and calculated parameters derived from linear regression of the Arrhenius-plot of the parameter α_w in CSM at 27% total solids.

27% TS	Slope $\mu_{\alpha_{w,T}}$ [K · s]	$log\ \alpha_{w,T_0}$ [s]	$\alpha_{w,T_{ref}}$ [s]	z-Value [K]	E_a [kJ mol^{-1}]	Q_{10}
β_w variable, non-preheated	9268.2	-21.26	362.8	17.1	177.5	3.86
$\beta_w = 1.5$, non-preheated	8481.4	-19.14	336.7	18.8	161.4	3.40
$\beta_w = 1.5$, preheated	5475.3	-11.23	694.4	29.8	104.8	2.17

Tab. 7–2: Parameters and calculated parameters derived from linear regression of the plot of the parameter α_w depending on total solids content of CSM at a heating temperature of 116 °C.

116°C	Slope $\mu_{\alpha_{w,s}}$ [K · s]	$log\ \alpha_{w,s_0}$ [s]	$\alpha_{w,s_{ref}}$ [s]
β_w variable, non-preheated	-0.0764	4.455	246.9
$\beta_w = 1.5$, non-preheated	-0.0715	4.395	292.0

Compared to *z*-values of the inactivation of spores of pathogenic microorganisms in the range of 10-12 in most cases and up to 19 K in some cases (van Asselt and Zwietering 2006), these z-values for protein aggregation indicate that higher heating temperatures combined with shorter holding times will achieve higher *log*-reduction concerning microbial inactivation while limiting aggregation of casein micelles. When skim milk was UHT preheated prior to concentration as described, the temperature dependency decreased resulting in a higher *z*-value and lower activation energy in the range of chemical reactions as shown in Fig. 7-4a.

Prediction of heat-induced coagulation by secondary modelling

The regression parameters were then used to calculate the relationship of heating temperature, heating time, total solids content, and the expected amount of sediment (four dimensions) based on the mean of the parameter estimates. Then, certain criteria like heating temperature-time-relationships necessary for microbial inactivation can be defined and the possible working range for heat treatment can be estimated based on a certain tolerable amount of aggregated protein, e.g. maximum 2% of aggregated protein.

At first, we wanted to show the modelling results for the course of sediment formation, i.e. heat-induced aggregation depending on total solids in Fig. 7-5a and depending on heating temperature Fig. 7-5b at a constant CSM total solids content of 27% including extrapolated data (*dotted lines*) on a semi-logarithmic scale. The lower boundary $\frac{C_\infty}{C_0} = 0.48$ for ≥ 18% CSM total solids and all heating temperatures was used which means that after completion of the coagulation process, about 48% of caseins and whey proteins had become soluble. A critical level of 2% of sedimentable protein of total protein is indicated (*dashed lines*). This prediction shows that reducing the critical level of sedimentable protein to much lower acceptable will largely affect the maximum holding time possible. By contrast, increasing the critical levels to 5% doubles the holding time possible for heat treatment compared to a critical level of 2%. Changes in possible holding times will become increasingly smaller with increasing relative amount of tolerated aggregated protein.

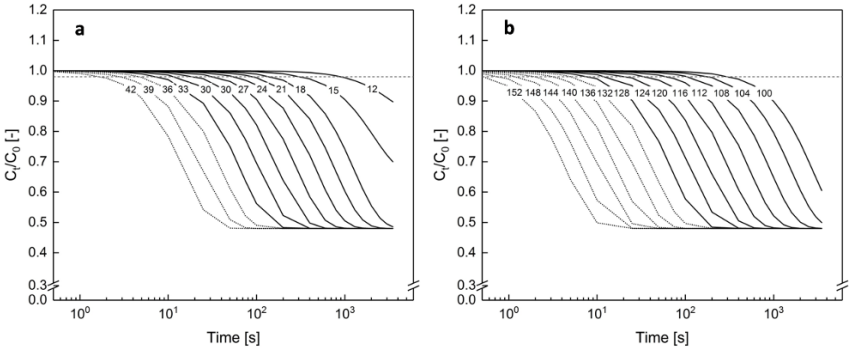

Fig. 7-5: Simulation of the relative amount of non-sedimentable protein depending on CSM total solids at 116 °C (a) and 27% CSM total solids depending on heating temperature over time on a logarithmic scale (b). A critical level of 2% of sedimentable protein of total protein indicates the onset of coagulation *(dashed lines)*. Calculations based on extrapolated data are also shown (*dotted lines*).

Predictions based on the model parameters for a critical level of 2% of sedimentable protein depending on heating temperature, holding time, and CSM total solids are shown in Fig. 7-6. Certain microbial inactivation levels were also included in Fig. 7-6 which are, strictly speaking, only valid for unconcentrated milk or other media (van Asselt and Zwietering 2006).

Due to the z-value of heat-induced aggregation of casein micelles of about 18 K, direct heat treatment by injection as used by Dumpler and Kulozik (2016) or infusion is very promising to maximize product quality and safety of heat sensitive products using very short holding times < 1 s. Modelling in this study and comparison with experiments of Dumpler and Kulozik (2016) at pilot scale was very instructive as discrepancies in possible holding times of less than 6 s were found concerning the onset of coagulation that could be caused by residence time distribution phenomena. However, isothermal static indirect heat treatment is impossible in this range of temperature and holding times >130°C/< 2 s even when smaller times would be used.

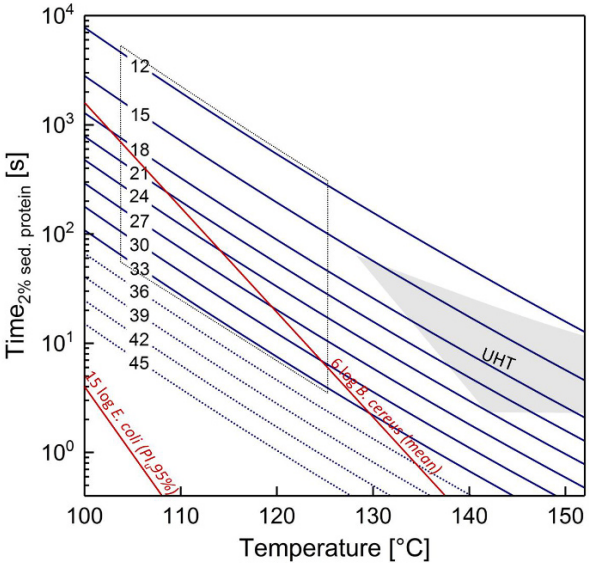

Fig. 7-6: Iso-effect lines for CSM for a critical level of 2% of sedimentable protein of total protein depending on heating temperature, heating time, and total solids content of bulk CSM. The time-temperature range covered by experiments *(dotted area)*, extrapolated total solids contents (*dotted lines*) are shown. The UHT region *(grey area)* and certain microbial inactivation levels are indicated for orientation.

Dissociation and aggregation of individual caseins

In the previous paragraph, we referred to the dissociation of caseins from casein micelles that become non-sedimentable as they dissociate into the serum phase and form colloidal particles as shown by Dumpler et al. (2017c) either during heating or after cooling as assumed by Anema and Li (2000). The relevance of dissociated protein is, on the one hand, linked to the relative amount of protein present in the serum phase after complete coagulation, i.e. the lower asymptote of the Weibullian model, and on the other hand, heat-induced coagulation which is often related to dissociation of κ-casein as one of the main detrimental effects on casein micelles' colloidal

stability. Using direct steam injection, dissociation of κ-casein at the onset of coagulation was also found to be comparably low by Dumpler et al. (2017c). In the study of Dumpler et al. (2017c), κ-casein was not included in protein aggregates together with calcium sensitive caseins to a large extent. The reason for the lower amount of coagulable protein in CSM using the tube heating system as compared to direct steam injection should be clarified. Hence, we wanted to investigate the distribution of caseins depending on heating time at different total solids and heating temperatures.

A selection of data, including κ-casein and α_{s1}-casein as examples, is shown in Fig. 7-7. The decrease in the amount of detectable κ-casein and α_{s1}-casein in the centrifugal supernatant at 4,000*x*g/10 min depending on heating time for a constant total solids content of 27% varying heating temperature is shown in Fig. 7-7a and Fig. 7-7c. Compared to α_{s1}-casein, κ-casein showed a relatively limited aggregation over time starting from the onset of coagulation. About 20-30% of κ-casein had coagulated together with calcium sensitive caseins. The extent of coagulation at a certain time of the two caseins was dependent on heating temperature and followed the general trend of protein aggregation. This was also observed for different CSM total solids heated at 116 °C (Fig. 7-7b and d).

In this dataset, we also included the data of the ultracentrifugal supernatant obtained at 48,800*x*g/26 min containing dissociated casein. We could neither observe a pronounced degree of dissociation of κ-casein at the onset of coagulation at different total solids nor a clear correlation between aggregated caseins and dissociated κ-casein which was in the range of 30-40% at the onset of coagulation. However, we have to state that the determination of the amount of caseins in heated CSM was complicated compared to the analysis after direct steam injection heat treatments as thermal degradation due to crosslinking, Maillard type reactions, and thermal breakdown were pronounced. This could be observed for α_{s1}-casein at holding times > 1,000 s (Fig. 7-7d) and especially for κ-casein (Fig. 7-7b).

The determination of total nitrogen after centrifugation was, however, a more robust procedure to follow heat-induced aggregation as it is less influenced by chemical reactions. Nevertheless, we can state that after about 1,600 s of heating at 116 °C, when coagulation was complete for 18, 24, and 30% total solids, about 53% of κ-casein, 27% of α_{s2}-casein, 24% of α_{s1}-casein, and 36% of β-casein had dissociated from casein micelles. When we take into account a relative distribution of 12, 9.5, 36.5, and 42% of κ-casein, α_{s2}-casein, α_{s1}-casein, and β-casein, respectively, and assume the denatured whey proteins to be present in the serum phase to about 70%, we can calculate a relative amount of serum protein to be 47% which is very close to the observed total protein.

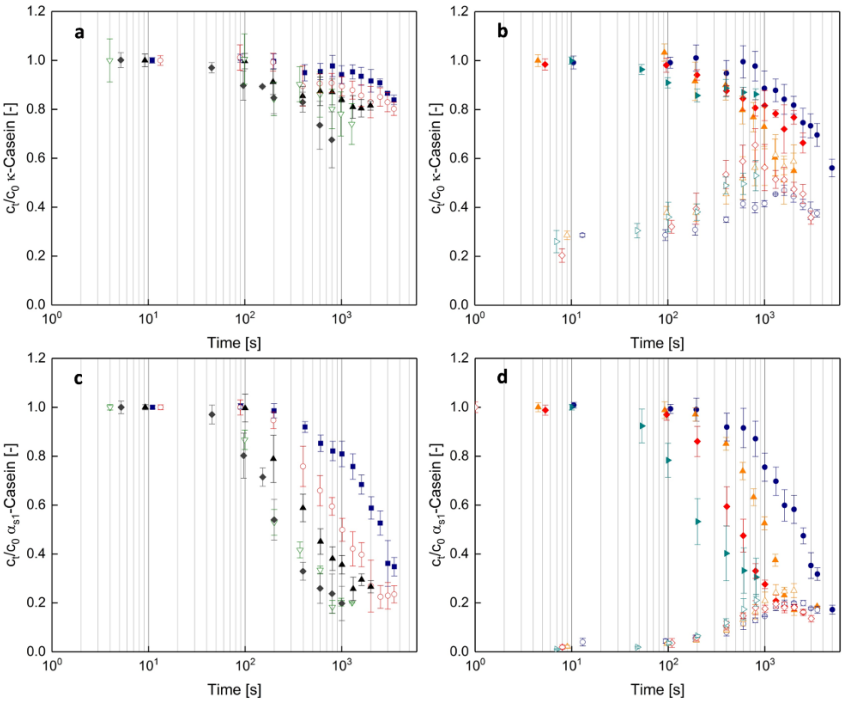

Fig. 7-7: Relative amount of soluble κ-casein (a) and α_{S1}-casein (c) depending on heating temperature at 27% CSM total solids over time at 4.000*x*g/10 min. Heating temperatures were 104 (■), 108 (○), 112 (▲), 116 (▽), and 120 °C (◆).The amounts of κ -casein (b) and α_{S1}-casein (d) including soluble protein in the ultracentrifugal supernatant *(open symbols)* at 48.800*x*g/26 min is shown depending on CSM total solids of 12 (●,○), 18 (◆,◇), 24 (▲ ,△), and 30% (▶ , ▷) at a constant heating temperature of 116 °C.

7.4 Conclusions

In this study, a first attempt to kinetic modelling of heat-induced coagulation of concentrated milk using a Weibullian model was made. We propose that heat-induced coagulation is a two-step process. It includes a destabilisation phase of casein micelles including κ-casein dissociation and other factors affecting micelle stability such as an increase in size of casein micelles that are influenced by milieu and heating conditions. This is then followed by an aggregation phase where again certain milieu conditions affect the extent of coagulation and the shape of the aggregates formed whereby continuous destabilisation of remaining micelles proceeds. According to the Weibullian model, the rate of aggregation of casein micelles is increased as $\beta_w > 1$ was estimated which means that the hazard function is increasing over time due to

ongoing destabilisation of non-aggregated casein micelles. This model is capable to summarize the overall heat-induced effects, be they either adverse like polymerisation of caseins and low calcium solubility at high temperature or conducive like changes in pH, hydrolysis of caseins, and hydrolysis of phosphoserine residues to coagulation of caseins. In addition, the data presented are dependent on the overall heat stability of the milk used and this should be taken into account. An integration of technological measures to increase heat stability like preheating of milk could be modelled as well. Despite the use of a Weibullian model for heat-induced coagulation, an integrated approach for optimisation of processing parameters using classical n-*th* order kinetics for microbial inactivation and chemical reactions is possible. Data on the heat-resistance of microorganisms and spores in concentrated milk need to be further addressed.

Appendix

The plot of residuals for the Weibullian model (eq. 2) used in Fig. 7-1 for fitting the obtained normalized data using $\beta_w = 1.5$ as constant is shown.

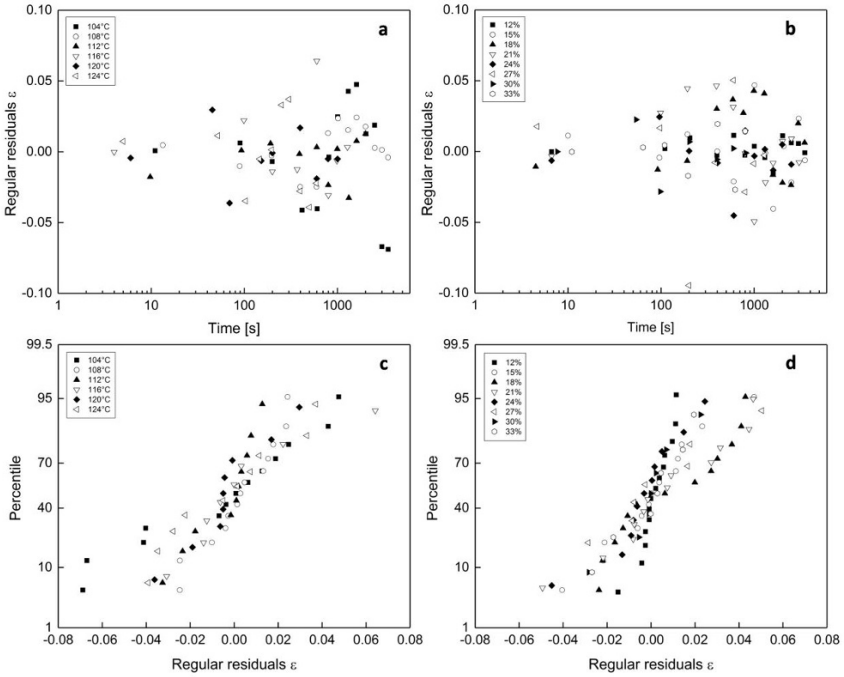

Fig. 7-8: Regular residuals of the Weibullian model fitted to the data of relative amounts of sedimentable protein of total protein for the dataset of different temperatures at a constant CSM total solids content of 27% (a) and at 116°C heating temperature for different total solids content (b). Panels (c) and (d) show the normal probability plots of the residuals.

A Kolmogorov-Smirnov test showed that residuals are consistent with a normal distribution (P = 0.39) where the normal distribution has a mean of 0.003342 and standard deviation of 0.02297 for the trials with 27% CSM total solids and varying heating temperature (n = 50). One outlier was identified. For the Kolmogorov-Smirnov test using data obtained for different skim milk total solids heated at 116 °C, the test showed that residuals are consistent with a normal distribution (P = 0.15) where the normal distribution has a mean of 0.0045593 and standard deviation of 0.01705 (n = 78). Six outliers had to be removed (some data from 12 and 33% total solids) to obtain normally distributed residuals.

Summary and contribution of the doctoral candidate

Modelling approaches to predict the onset of coagulation over a broad range of heating temperatures, heating times, and total solids contents could help to resolve the conflict between microbial inactivation and heat-induced aggregation of casein micelles in concentrated skim milk (CSM). If formal reaction kinetics applies for the heat-induced aggregation of casein micelles in CSM, non-isothermal heat treatments could also be described concerning their effect on the heat-induced coagulation of heated CSM.

Therefore, the aim of this study was to estimate the heat stability of CSM of various total solids content by a quantitative investigation of the heat coagulation process of proteins in CSM. The hypothesis was that the heat coagulation process follows kinetics and can be described by a mathematical model in dependency of total solids, heating temperature, and heating time. The amount of sediment formed for any concentrate at any temperature and holding time should be made calculable using secondary modelling.

The results of this study showed that critical levels of heat-induced destabilisation of concentrated milk could be defined. A quantitative definition of the working ranges in terms of temperature-time combinations possible for the heat treatment of concentrated skim milk of a certain total solids content to achieve sufficient microbial inactivation without coagulation were given. The results were supposed to contribute to a significant extent to the field of research on the heat stability of casein micelles in concentrated milk systems.

The major contributions of the doctoral candidate were the design of experiments, sample preparation procedures, and the evaluation of the data obtained. Next to this, the doctoral candidate carried out data analysis and worked out the proposed model to calculate heat stability of concentrated skim milk. The experiments were designed and the manuscript was written by the doctoral candidate after iterative critical review of the existing literature.

8 The effect of calcium and pH on heat treated micellar casein[11]

Abstract

Changes in micelle size observed by particle sizing techniques in micellar casein concentrate as an indication of weakening of the structure of casein micelles were investigated. The changes observed were related to heat-induced dissociation and coagulation of casein micelles depending on milieu conditions characterized by pH, calcium activity, ionic strength, and dissociated casein. It could be shown that, in contrast to dissociation of caseins, the increase in the hydrodynamic radius of casein micelles was closely related to heat-induced coagulation of casein micelles. Dissociation of caseins was found to increase with increasing pH and lower soluble calcium concentration. In addition, low ionic strength in the serum resulted in gel formation of destabilised micelles, whereas at high ionic strength, the formation of distinct large particles could be observed. We therefore concluded that dissociation of caseins from micelles and the coagulation of casein micelles in whey protein free milk systems are opposite reaction paths both destabilising the micellar structure. The coagulation of casein micelles is the subsequent reaction of changes that occur upon heating of casein micelles only under certain milieu conditions. The definition of heat stability as equivalent to heat-induced coagulation therefore needs to be reconsidered.

11 Original publication: Dumpler J, Dörle M, Kulozik U (2017) Implication of pH and soluble calcium on micelle size and dissociation of κ-casein in relation to heat stability of micellar casein. **Submitted** to LWT – Food Science and Technology. Adapted original manuscript. Adaptions of the manuscript refer to title, enumeration type, citation spelling, notation of units, and format.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_8

Highlights

- Micellar casein concentrate (MCC) was used to investigate heat stability of micelles
- Dissociation of caseins in MCC was not related to coagulation of casein micelles
- A relationship between ionic calcium, pH, and micelle size in heated MCC is proposed
- Changes in micelles size are supposed to be related to heat-induced coagulation of MCC

8.1 Introduction

Minerals inside casein micelles are known to be in equilibrium with the milk salt composition of the serum phase in milk. These micelles withstand severe heat treatments due to inner stabilisation by calcium bridges and calcium phosphate and their colloidal structure stabilised by κ-casein (Holt and Horne 1996; Singh 2004; Dalgleish 2011; Holt et al. 2013). Factors that affect the colloidal stability of casein micelles can be divided in three major categories.

Firstly, chemical changes including thermal degradation of amino acids and casein polypeptide chains, cleavage of phosphoserine residues (Aoki et al. 1990), and cleavage or inter- and intramolecular covalent crosslinking of polypeptide chains (Lorient 1979; Bulca et al. 2016). Further chemical reactions include Maillard-type reactions, precipitation of calcium phosphate onto casein micelles or within the serum phase (Rose 1962), and degradation of lactose which leads to a coincident decrease in pH during heating (van Boekel et al. 1989a).

Secondly, technological pre-treatments of the casein micelles by enzymatic crosslinking or cleavage of phosphoserines or peptide bonds, preheating of milk, high shear forces, high hydrostatic pressure treatments, extensive cold storage, removal of colloidal calcium phosphate (CCP), concentration, removal of whey proteins will affect heat stability (Fox and Hoynes 1975; Muir 1984; Holt et al. 1986; Huppertz et al. 2004; Huppertz 2014).

Thirdly, milieu conditions prior to heat treatment, i.e. initial pH, ionic strength, soluble calcium, calcium-to-phosphate/citrate ratio, and the presence of whey proteins, will affect changes on casein micelles during heating (Rose 1961a; Fox and Hoynes 1975; Singh et al. 2007). These changes can either lead to excessive coagulum formation during heat treatment or excessive dissociation of casein. The former case is called heat-induced instability observable as large flocs, gels or large sedimentable particles in the subjective heat stability test. The latter process was considered as heat stable although structural changes on casein micelles due to dissociation of caseins under certain milieu conditions without coagulation might be at least as severe as in the case of coagulation.

During membrane processing, diafiltration is often applied to remove milk serum constituents by addition of water of different mineral composition. Reverse osmosis permeate or tap water are used to reduce the amount of lactose and soluble salts in the casein fraction. Thereby, the protein content of the final milk protein concentrate (MPC) is increased. Excessive diafiltration is necessary to remove a large proportion of whey proteins due to higher rejection of whey proteins compared to soluble low molecular weight components (Kulozik and Kersten 2002; Tolkach and Kulozik 2006; Hurt et al. 2010).

After fractionation, heat treatment is applied to improve microbial quality of the final powder (Stoeckel et al. 2014). Heat stability of casein micelles in diafiltered milk can vary widely and heating intensities as low as pasteurisation might be sufficient to induce severe coagulation. Extensive changes in heat stability of casein micelles due to changes in serum composition, i.e. changes in the calcium-to-phosphate ratio, pH, ionic strength, and non-protein nitrogen can lead to changes in micelle hydration and dissociation. These changes induce dissociation of caseins and/or severe coagulation of casein micelles during heating depending on the extent of change (Hurt and Barbano 2015) as well as on the intensity of heat treatment (Kelly et al. 2000; Crowley et al. 2014; Crowley et al. 2015) as compared to milk. The disruption of the colloidal state by coagulation of caseins was taken as an indicator for low heat stability. A high amount of dissociated casein was not attributed to an unstable state. Interestingly, dissociation of caseins was assumed to be a reaction step necessarily preceding coagulation, although a clear relationship between changes in milieu conditions, the amount of dissociated casein, and the onset of coagulation has not been established to date. Heat-induced dissociation of κ-casein was assumed as a prerequisite for heat-induced coagulation. However, heat-induced dissociation also in the absence of whey proteins at pH > 6.7 was found to be high and coincident with a high heat stability, i.e. retarded coagulation (Singh and Fox 1987c). In addition, changes in pH on heat stability, for example, could not in all cases be attributed to individual components. Changes in pH will affect multiple components in milk, i.e. milk salts, whey proteins, and caseins, at the same time.

One of the observations that is likely to be linked to coagulation is the marked increase in hydrodynamic radius observed in milk and concentrated milk heated at pH < 6.7 (O'Connell and Fox 2000; Mounsey and O'Kennedy 2009; Taterka and Castillo 2015; Dumpler and Kulozik 2016; Dumpler et al. 2017c). A relation of these changes to heat stability of casein micelles has not yet been established. However, the increase in size upon heat treatment of whey protein free casein dispersions was limited at pH > 6.7. Coincident, a higher heat stability was observed by Sauer and Moraru (2012). A correlation between the increased hydration of casein micelles, leading to a tremendous increase in calcium sensitive and hydrophobic surface of micelles, and the relation to coagulation remained uncertain. The effect of soluble calcium on both, micelle size and κ-casein dissociation, in combination with initial pH and ionic strength and their implication on heat-induced coagulation of casein micelles has not been investigated. It is not clear if dissociation of caseins and the increase in hydro-

dynamic radius are two different effects destabilising casein micelles, whereby only the latter possibly leads to coagulation.

From a scientific point of view, casein micelle isolates have been prepared to study the heat-induced changes of casein micelles in a less complex environment than the multi-component system of milk or concentrated milk (Aoki et al. 1974; Fox and Hoynes 1975; Aoki et al. 1975; Aoki et al. 1977; Mounsey and O'Kennedy 2009). Effects of single changes in the milieu of casein micelles can then be investigated. These 'native' milk protein isolates or micellar caseins were mostly prepared by dialysis against simulated milk ultrafiltrate (SMUF), micro- or ultrafiltration including diafiltration or ultracentrifugation and re-suspending of the pellet in ultrafiltration permeate or SMUF (Singh and Fox 1987c). These simplified milk-like systems have been studied to a much lesser extent. However, the industrial relevance of such studies has increased since such micellar casein concentrates are now if commercial interest.

Therefore, the aim of this study was to investigate the effects of the soluble calcium on overall heat stability of micellar casein as a model system for other liquid milk products. In addition, the effects of initial pH before heating and after heat treatment, dissociation of caseins, ionic strength, calcium activity, and casein micelle hydrodynamic radii depending on heating intensity in relation to heat-induced coagulation of micellar casein has been investigated. Thereby, the destabilising mechanisms leading to excessive coagulation of casein micelles were assessed.

8.2 Materials an methods

Manufacture of micellar casein concentrate and subsequent spray drying

Pasteurised (74 °C/28 s) skim milk was obtained from a local dairy factory (Molkerei Weihenstephan, Freising, Germany). A batch of 300 L of skim milk was preheated to 50 °C and concentrated by a factor of 2.5 at 50-52° C to increase diafiltration efficiency using 1.4 µm ceramic gradient membranes (TAMI Isoflux® Sunflower, TAMI Germany GmbH, Hermsdorf, Germany) in a microfiltration (MF) pilot plant. Eight subsequent diafiltration steps were performed using 960 L of simulated milk ultrafiltrate (SMUF) for a working temperature of 50 °C as described by Dumpler et al. (2017a) to remove whey proteins, lactose and non-protein nitrogen components as interfering substances during heat treatment of liquid micellar casein concentrates (MCC). At the same time, the mineral salt equilibrium in skim milk was maintained to avoid dissociation and aggregation reactions during filtration which was observed when distilled water was used as a diafiltration medium due to changes in the mineral equilibrium of milk (Ferrer et al. 2014). One diafiltration step is defined as the amount of diafiltration medium required to exchange the amount of serum present in the MF retentate. Diafiltration with lactose-free 'SMUF 50 °C' was performed in a continuous mode. Average transmembrane pressure was 1.2 bar; the pressure drop across the membrane length was 1.5 bar. After diafiltration, the MF retentate was further concentrated by a factor of 3.6 to obtain an approximate casein concentration

of 10%. Fig. 8-1 shows the amount of major whey proteins present in skim milk, MCC after concentration prior to diafiltration (1), and diafiltration (2-8). The average permeation of whey proteins was 50%. This means that in the permeate, the concentration was half the concentration of whey proteins in the retentate fraction at all times. The concentrate was cooled to 4 °C and stored overnight. The process block flow chart is also included in Fig. 8-1. After preheating to 40 °C, the MCC was spray dried in a co-current spray drier (PRODUCTION MINOR™, GEA Niro, Søborg, Denmark) using a rotary disc at 15,000 rpm. Air inlet temperature was 190 °C and the air outlet temperature was kept at 78-80 °C. The MCC powder was cooled, packed in air tight double-sealed polyethylene bags and stored in the dark at ambient conditions.

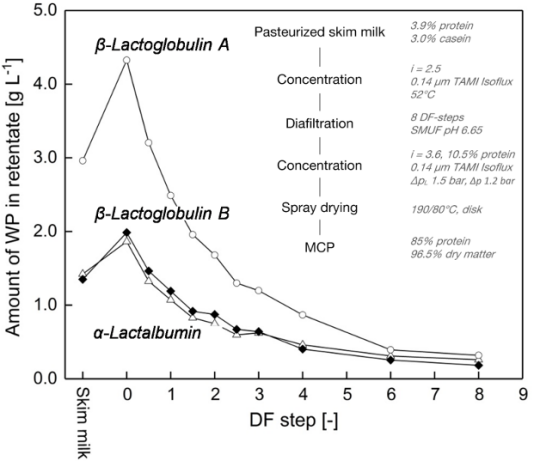

Fig. 8-1: Amount of whey proteins in the microfiltration (MF) retentate of during concentration and diafiltration (DF) of milk with simulated milk ultrafiltrate. The block flow chart of the process of micellar casein concentrate (MCC) powder is included.

Powder reconstitution and sample preparation

The composition of the MCC powder is shown in Tab. 8–1. The MCC powder was reconstituted by combining 14.5 g powder with 100 mL of distilled water to achieve approximately the same dry matter as before drying. The mixture was stirred at room temperature for 30 min. The reconstituted MCC was subsequently chilled on ice and stored at 4 °C overnight for rehydration. This mixture was then heated to 52 °C in a water bath for 30 min, degassed at 150-200 mbar, and cooled to room temperature. Thereafter, dry matter was determined and adjusted to 12.4% by addition of a small amount of distilled water. Addition of soluble calcium was performed using a 10% (w/v) calcium chloride solution. Addition of potassium chloride was performed using a 3.5 M KCl solution. Subsequent adjustment of pH in MCC was performed by dropwise addition of 4 and 2 M HCl or 2 M NaOH under vigorous stir-

ring using a magnetic stirrer in a beaker. Dilution of the MCC in terms of protein content by addition of salt solutions and pH-adjustment was no more than 5%.

Tab. 8–1:Composition of the MCC powder.

Analysis	
Dry matter [%][b]	96.5 ± 0.03
Protein [%][b]	83.7 ± 0.27
Protein (dry basis) [%]	86.4 ± 0.27
Lactose [g/g][a]	-

[a]not detected
[b]n = 3

Measurement of dry matter, density, protein, pH, and calcium activity
Dry matter of the liquid MCC and MCC powder was determined using a microwave drying system (CEM Smart Turbo 5, CEM, Kamp-Lintfort, Germany). Density of the liquid MCC was determined using a density meter DMA 4100M (Anton Paar, Graz, Austria). Protein content of the liquid MCC and MCC powder was determined as described by Dumpler and Kulozik (2016) according to the method of Dumas using a vario MAX cube (Elementar Analysensysteme GmbH, Hanau, Germany). A nitrogen conversion factor of 6.25 calculated from the distribution of individual caseins within the MCC based on RP-HPLC and the amino acid sequence of individual caseins was used. The pH of the reconstituted MCC and diluted MCC was determined using a pH meter Multical pH 526 (WTW, Weilhein, Germany). Calcium activity was determined a described by Dumpler et al. (2017a) using a perfectION™ calcium selective combination electrode (Mettler-Toledo AG Analytical) connected to the ALMEMO® 2590-4AS via a ZA9000 FS2 adapter cable (Ahlborn Mess- und Regelungstechnik, Holzkirchen, Germany). Calcium chloride solutions of different concentration in the range of 0.18 to 146.7 mM Ca^{2+} prepared from a 3.886 M calcium chloride standard solution were used for calibration of the electrode. Calibration and measurements were performed at 25 °C. Measurements of calcium activity in MCC were performed in quadruplicate.

Heat treatment and centrifugal separation of heated MCC
After adjustment of pH, ionic strength, and calcium content, MCC samples were heat treated at different holding times and temperatures as described by Bulca et al. (2016) and Dumpler et al. (2017b). In short, samples were filled into 15 mL stainless steel tubes with an inner diameter of 8 mm, tightly screwed and heat treated by indirect heating with saturated steam and subsequently cooled by cold tap water. After rapid cooling to room temperature, samples were gravimetrically diluted 1:3.5 with 'SMUF 50 °C' (pH 6.65) that had also been used for diafiltration. The adoption of the pH of the SMUF to every single sample pH after heat treatment was not feasible and therefore, this change in pH needs to be taken into account for correct interpretation of the results in this study.

Dilution was calculated by taking into account the density of the SMUF solution and the micellar casein. Diluted samples were then aliquoted and centrifuged at 4,000*x*g/10 min in 50 mL screw capped Falcon tubes for separation of heat-induced casein aggregates. The supernatant was removed and part of it further centrifuged at 48,800*x*g/26 min as described by Dumpler et al. (2017c). Diluted samples, centrifugal, and ultracentrifugal supernatant were then further analysed by RP-HPLC and particle sizing techniques. Two samples were heat treated in each trial and trials were performed in duplicate (n = 4).

RP-HPLC analysis of MCC and centrifugal supernatants

The liquid MCC before spray drying and the reconstituted MCC were analysed for major caseins κ-casein, α_{S2}-casein, α_{S1}-casein, β-casein, and the major whey proteins α-lactalbumin, β-lactoglobulin B, β-lactoglobulin A as described by Dumpler et al. (2017c). Physical characteristics and protein composition of the MCC prior to drying are shown in Tab. 8–**2**. Centrifugal and ultracentrifugal supernatants were prepared for reversed-phase high performance liquid chromatography (RP-HPLC) as also described by Dumpler et al. (2017c). The major caseins κ-casein, α_{S2}-casein, α_{S1}-casein, and β-casein were separated using the gradient as described by (Dumpler et al. 2017b).Data in this study are reported as relative amounts of individual caseins compared to the unheated samples. Relative amounts of caseins in the supernatant were corrected for thermal degradation due to thermal crosslinking and other chemical reactions of caseins. The decrease in detectable caseins by RP-HPLC in the centrifugal and ultracentrifugal supernatant was integrated by referring the soluble amount of casein to the detectable amount of casein at heating time *t* by eq. 8.1.

$$\frac{c_t}{c_0} = \frac{c_{t,z}}{c_{t,total}} \tag{8.1}$$

whereby c_t is the concentration of an individual casein in the centrifugal or ultracentrifugal supernatant at time t corrected by thermal degradation, c_0 is the concentration of a casein in the unheated sample, $c_{t,z}$ is the concentration at time t in the supernatant, and $c_{t,total}$ is concentration of a casein in the heated uncentrifuged sample at time t. Thereby, it was assumed that thermal degradation of caseins in MCC is independent of the state of caseins within the heated MCC, i.e. whether contained in or dissociated from casein micelles. Each sample heat treated at a certain temperature and heating time was analysed as single determination (n = 4).

Tab. 8–2:Physical characteristics and protein composition of the reconstituted MCC.

MCC/reconstituted MCC	
Dry matter [%][a]	12.45 ± 0.03
pH [-][a]	6.8 ± 0.02
Protein [%][b]	10.12 ± 0.06
Density [g cm^{-3}]	1.038
Amount [g L^{-1}][b]	
κ-Casein	14.55 ± 0.38
α_{S2}-Casein	9.40 ± 0.39
α_{S1}-Casein	33.85 ± 0.86
β-Casein	38.15 ± 0.72
Sum of Caseins	95.95
α-Lactalbumin	0.44 ± 0.01
β-Lactoglobulin B	0.38 ± 0.01
β-Lactoglobulin A	0.49 ± 0.03
Sum of whey proteins	1.31

[a]n = 15
[b]n = 3

Determination of heat stability of MCC on laboratory scale

Heat stability of MCC before and after spray drying was determined as described by Dumpler and Kulozik (2015). In short, 10 mL of sample were filled in a 50 mL screw capped centrifuge tube including a Pt-100 temperature sensor to record the sample temperature and tightly screwed. The filled tubes were then immersed in an oil bath adjusted to 155 °C. Heat coagulation temperature and time were recorded at the onset of visible coagulation of the samples. Measurements were performed in triplicate. This subjective heat stability test was also performed for MCC depending on soluble calcium addition under variation of the initial pH of the samples in the range of pH 6.4-7.2. Results after spray drying as compared to the MCC before spray drying in terms of heat coagulation time and temperature were similar.

Determination of particle size by laser light diffraction and photon correlation spectroscopy

Heated MCC, centrifugal and ultracentrifugal supernatants were analysed by photon correlation spectroscopy (PCS) at 20 °C as also described by Dumpler and Kulozik (2016). Prior to measurements, samples were diluted 1:20 with SMUF 50 °C and filtered through a 1.0 µm syringe filter (Chromafil® GF-100/25, Macherey-Nagel, Düren, Germany) for centrifugal supernatants and a 0.2 µm (Chromafil® Xtra RC-20/25, Macherey-Nagel, Düren, Germany) syringe filter for ultracentrifugal supernatants to remove dust as interfering particles with measurements.

Data analysis
For data analysis of RP-HPLC chromatograms an Agilent ChemStation software (Rev. B.04.03) was used. Data was plotted using OriginPro 2017 (OriginLab Corporation, Northampton, MA, USA).

8.3 Results and discussion

Changes in pH during heat treatment and dilution of MCC
The decrease in pH in MCC over time depending on heating temperature is shown in Fig. 8-2a. Compared to the results obtained in unconcentrated milk and concentrated milk described in literature (van Boekel et al. 1989a), we found that the decrease in pH in MCC was not very pronounced. However, the increase in slopes with increasing temperature, i.e. the rate constants, indicates chemical reactions to contribute mostly to heat-induced acidification in MCC. An approximate *z*-value for the decrease in pH was found to be around 30 K. All pH-values within the temperature-time range investigated were pH > 6.5 which is the range of relatively high heat stability as shown later.

A decrease in pH due to chemical reactions during heat treatment was often related to coagulation of milk due to the reduction of the net negative charge of casein micelles. This would, however, mean a higher heat stability of MCC without lactose to be expected over the entire pH range compared to milk or concentrated milk. This was found to be not the case as aldehydes will also induce crosslinking of caseins (Fox and Hearn 1978b; Holt et al. 1978b). Thermal degradation of lactose to formiate and Maillard-type reactions (1) significantly contribute to the decrease in pH besides precipitation of calcium phosphate (2), cleavage of phosphoserine residues (3) (van Boekel et al. 1989a). In our opinion, thermal hydrolysis of peptide chains (4) and deamidation reactions (5) might also significantly contribute to heat-induced acidification. In MCC, reaction (1) can be excluded which accounts for about 50% to the decrease in pH in milk (Singh 2004).

Changes in pH after pH-adjustment were approximately linear and the same slope of the curves was found at a heating temperature of 116 °C. Addition of calcium chloride lowered the initial pH resulting in a pH of 6.5 at 3 mM added calcium chloride. The decrease in pH was again similar (data not shown). We therefore consider the contribution of the change in pH during heating to coagulation of caseins in MCC with an initial pH > 6.5 as a minor effect.

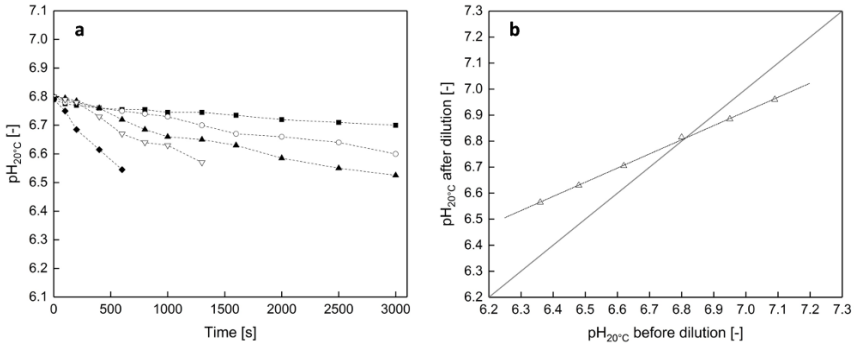

Fig. 8-2: Decrease in pH during heat treatment of MCC at different temperatures (a) and the corresponding pH after dilution of MCC with SMUF buffer pH 6.65 after dilution and storage for 24 h at 4 °C (b).

Due to the necessity of complete removal of heat-induced aggregates from MCC, a dilution step before centrifugation was included in the sample preparation procedure. The resulting pH after dilution and equilibration of 12 h of heated samples with SMUF pH 6.65 is shown in Fig. 8-2b. It can be seen that the dilution led to an increase in pH for samples with pH < 6.8 and a decrease in pH for samples with pH > 6.8.

Heat-induced dissociation of caseins in MCC depending on temperature

Heat-induced dissociation of casein monomers or as particles from casein micelles in skim milk, concentrated skim milk and whey protein-free casein micellar casein prepared by different techniques has already been studied to some extent. This was mostly done to find a correlation of heat stability of casein micelles and initial pH (Fox and Hoynes 1975; Fox and Hearn 1978a; O'Connell and Fox 2000). A variation of heating temperature at a fixed heating time was used and in most studies in literature. Milieu conditions in terms of initial pH and whey protein content in the serum phase were varied in these studies. It was found that the relative amount of κ-casein dissociated from casein micelles increases with increasing heating temperature and pH in milk containing whey proteins (Singh and Fox 1987c; Anema and Klostermeyer 1997; Anema and Li 2000, 2003b). Studies on the kinetics, i.e. the dissociation at a fixed temperature over time for individual caseins, were performed to a much lesser extent.

Hence, we wanted to investigate the course of dissociation and possibly coagulation of casein micelles in micellar casein over time depending on heating temperature. A quantitative relation of heat-induced destabilisation of casein micelles over time leading to coagulation depending on milieu conditions was intended. Fig. 8-3 shows that heating MCC with an initial of pH 6.8 at different temperatures resulted in an increased dissociation of individual caseins, especially κ-casein, with increasing temperature (Fig. 8-3a). The amount of dissociated calcium sensitive α_{S2}-casein, α_{S1}-casein, and β-casein indicated that the order of relative dissociation of individual

casein in MCC $\alpha_{S1} \geq \alpha_{S2} > \beta >> \kappa$ was similar to concentrated skim milk, inversely related to their calcium sensitivity, and might therefore hold for all other casein containing systems. The absolute amount of dissociated casein, of course, was highest for β-casein as it makes up to 40% of all caseins within the micelles. More than 70% of dissociated κ-casein could not be observed as samples heated at 140, 132, and 124 °C showed gel formation when this critical level was reached.

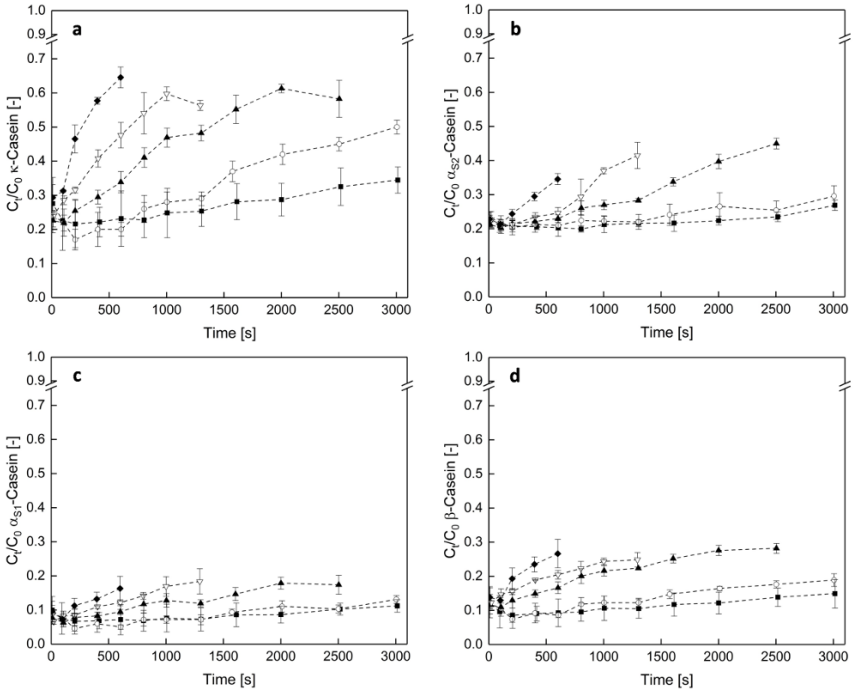

Fig. 8-3: Relative amount of dissociated κ-casein (a), α_{S2}-casein (b), α_{S1}-casein (c), and β-casein (d) analysed in the supernatant of MCC ultracentrifuged at 48,800*x*g/26 min after heating at temperatures of 108 °C (■), 116 °C (○), 124 °C (▲), 132 °C (▽), and 140 °C (◆) depending on heating time.

This indicates a possible relationship between κ-casein dissociation and heat-induced coagulation which led to gel formation in the case of MCC heated at high temperatures at pH 6.8. The expectation was, however, that coagulation of MCC would result in sedimentable particles as observed in concentrated skim milk by Dumpler and Kulozik (2015, 2016). The residual whey protein content in MCC that could lead to gel formation together with caseins was reduced to 1.31 g L^{-1}. Therefore, other factors contributing to heat-induced coagulation were likely to be responsible for whether gel or particle formation occurs. Possible other factors were the pH prior to heat treatment, the protein content, and the calcium content in the serum that could result

in changes related to casein micelles other than dissociation of caseins. Nevertheless, we can state that the heating temperature affects dissociation of caseins from casein micelle in MCC. The higher the heating temperature, the more dissociated casein can be found in the serum phase at the same heating time.

Heat-induced dissociation of caseins in MCC depending on pH

Following the hypothesis that dissociation of κ-casein causes coagulation, the dissociation over time was assumed to give an estimate on heat stability of casein micelles depending on milieu conditions. However, compared to milk and concentrated milk, whey protein free milk shows remarkably high heat stability, i.e. retarded coagulation at pH > 6.7 (Singh and Fox 1987c). This was found to be in conflict with the observation that at pH > 6.7, there was a strong increase in free soluble casein, especially κ-casein, after a heat treatment at a certain heating temperature when we assume a mechanistic relation between heat stability and dissociation of caseins (Anema and Li 2000; O'Connell and Fox 2000).

Therefore, MCC adjusted to different pH in the range of 6.35-7.1 and heat treated up to 3000 s at 116 °C to determine the amount of dissociated casein. The relative amounts of dissociated κ-casein and α_{S1}-casein are shown in Fig. 8-4a and b, respectively. The pH-dependency of α_{S2}-casein and β-casein dissociation over time was quantitatively similar to α_{S1}-casein. The relative amount of dissociated casein increased with increasing pH from 6.35 to 7.1 at 116 °C. At pH 7.1, no noticeable further increase in dissociated casein over time was observed in the ultracentrifugal supernatant. This observation could be related to the heat-induced decrease in pH and the dilution with SMUF of pH 6.65 which resulted in a pH of 6.8 in the sample heated for 3000 s at 116 °C with an initial pH of 7.1. Therefore, partial re-association of casein cannot be excluded.

Nonetheless, we can state that the adjustment of pH affected the dissociation of casein much more than the heat treatment, especially at pH > 6.8. Interestingly, ultracentrifugal supernatants of samples adjusted to pH > 6.8 without heating showed approximately half the amount of dissociated protein as compared to heated and immediately cooled samples (data not shown). This indicates that heating in addition to the adjustment of pH leads to a new equilibrium state by very fast kinetics compared to adjustment of the pH without heating. Despite this excessive dissociation of κ-casein, no coagulation of samples was observed, i.e. gel formation or sedimentable particles that could be separated at 4,000*x*g/10 min. Only the MCC adjusted to pH 6.35 before heat treatment at 116 °C showed slight heat-induced coagulation as shown later.

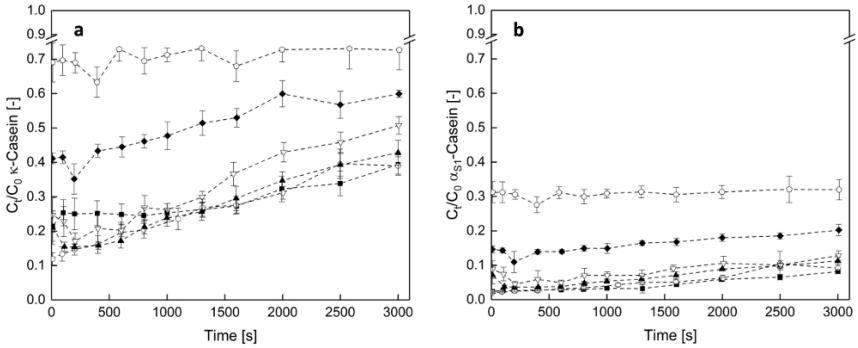

Fig. 8-4: Relative amount of dissociated κ-casein (a) and α_{S1}-casein (b) in MCC analysed in the supernatant of MCC ultracentrifuged at 48,800*x*g/26 min after heating at a temperature of 116 °C at pH 6.35 (■), 6.5 (○), 6.65 (▲), 6.8 (▽), 6.95 (◆), 7.1 (⬠) depending on heating time.

Relationship between heat stability of MCC, calcium activity and dissociation of caseins

The overall heat stability at natural pH of this model MCC prepared by diafiltration with SMUF at natural pH, the ionic strength of milk, very low whey protein content, and the ionic calcium level of unconcentrated milk was found to be high. A lower heat stability of MCC was expected from results in literature concerning the heat coagulation time of whey protein-free casein micelle dispersions with decreasing pH (Fox and Hoynes 1975; Singh and Fox 1987c). Another important aspect besides initial pH and heating temperature is the amount of soluble calcium present in the serum phase of milk products. Calcium is known to have a strong impact on casein micelles when heat treated (Sievanen et al. 2008; Faka et al. 2009; Omoarukhe et al. 2010; Ramasubramanian et al. 2012; Nian et al. 2012). Despite the general agreement in literature on the effects of soluble calcium on heat-induced coagulation of casein micelles in milk, little is known about the structural changes on casein micelles that occur after addition of soluble calcium during heat treatment and eventually lead to coagulation. The increase in soluble calcium besides the decrease in pH during concentration of milk by reverse osmosis or evaporation was assumed to be the major contributor to the decrease in heat stability with increasing total solids content. In addition to unknown structural changes on casein micelles, the amount of soluble calcium in the serum is linked to pH (Omoarukhe et al. 2010) and total solids content (Anema 2009; Omoarukhe et al. 2010). This makes it difficult to differentiate the effects of calcium and pH on casein micelle structure. We tried to achieve this by using a MCC as a simplified model system.

Fig. 8-5 shows the overall heat stability of MCC depending on pH in the range of pH 6.4 to 7.2 and addition of soluble calcium up to 3 mM as calcium chloride. Heat coagulation temperature Fig. 8-5a and the corresponding time Fig. 8-5b show that the heat stability of reconstituted MCC without the addition of soluble calcium drastically decreases at pH < 6.6. Heat stability of MCC was even lower when compared to

concentrated skim milk at the same casein content of approximately 35% (Dumpler and Kulozik 2015).

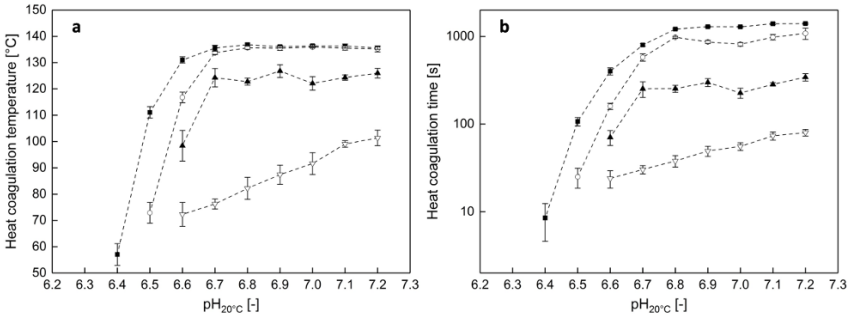

Fig. 8-5: Heat coagulation temperature (a) and heat coagulation time (b) of MCC depending on pH and addition of 0 (■), 1 (○), 2 (▲), and 3 mM (▽) soluble calcium as calcium chloride.

This is ascribed to the protective effect of denatured whey proteins in concentrated milk by steric stabilisation towards aggregation as also observed in MCC (data not shown). Addition of soluble calcium to MCC resulted in a pronounced decrease in heat stability even at high pH whereby the reduction of heat stability by additional adjustment of pH decreased. This indicates a close relationship of pH and soluble calcium influencing each other and thereby the heat stability of the casein micelles in MCC. However, it was not clear whether the decrease in pH increases calcium activity and thereby destabilises casein micelles or if the there is an additional effect of a reduced charge of casein micelles or other changes linked to micellar destabilisation.

Therefore, we determined the calcium activity in MCC depending on pH and calcium addition which is presented in Fig. 8-6 on a semi-logarithmic scale. It can be seen from the graphs that the calcium activity in MCC follows the general trend of heat stability of MCC, i.e. the calcium activity is in all cases higher at a lower pH and a higher amount of soluble calcium added to MCC. The measurable calcium activity is strongly affected by changes in pH. The relative amount of active calcium compared to the amount added to MCC is also affected by pH. At higher pH, a smaller portion of added calcium can be detected by the calcium selective electrode which is due to the lower solubility of calcium at higher pH and therefore the transition into the colloidal state as calcium phosphate. When we correlate this to the heat stability measurements, we can explain the smaller difference on terms of heat coagulation time and temperature at a higher pH of samples with different amounts of calcium addition (Fig. 8-5a and Fig. 8-5b). This strong impact of calcium and pH was expected to change the casein micelle structure so that the aggregation of casein micelles is facilitated. In the case of pH, we observed that the comparison between the dissociation of caseins (Fig. 8-4) and the results of the heat stability test (Fig. 8-5) showed no positive correlation, rather an inverse relationship.

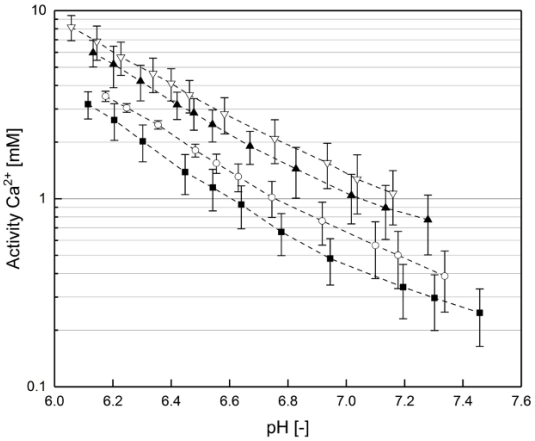

Fig. 8-6: Calcium activity in MCC depending on pH and addition of 0 (■), 1 (○), 2 (▲), and 3 mM (▽) calcium as calcium chloride.

The same was true for the dissociation of caseins depending on the addition of soluble calcium. Fig. 8-7a shows the relative amount of soluble κ-casein compared to overall κ-casein present in MCC. Fig. 8-7b shows the amount of soluble α_{S1}-casein in the ultracentrifugal supernatant of MCC heated at 116 °C without and with the addition of 1, 2, and 3 mM soluble calcium over time.

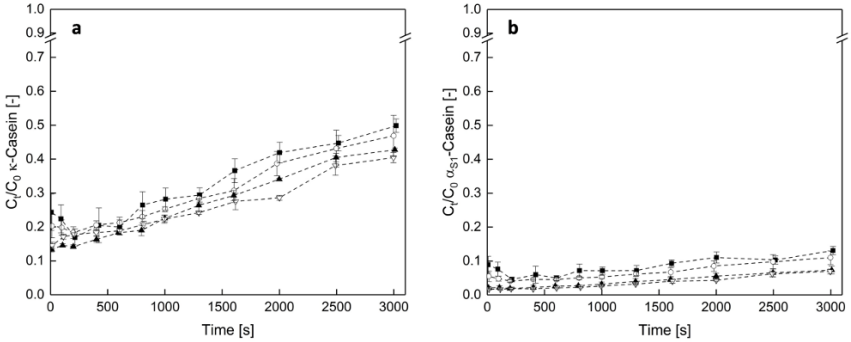

Fig. 8-7: Relative amount of dissociated κ-casein (a) and α_{S1}-casein (b) in MCC analysed in the ultracentrifugal supernatant of MCC centrifuged at 48,800*x*g/26 min after heating at a temperature of 116 °C and addition of 0 (■), 1 (○), 2 (▲), and 3 mM (▽) soluble calcium as calcium chloride.

The graphs show an increase in dissociated casein over time, the relative amounts for κ-casein being higher at all times compared to α_{S1}-casein. The graphs also show the tendency that with increasing calcium concentration the amount of dissociated casein is lowered at a certain holding time. Results for α_{S2}-casein and β-casein were

again similar to the results of α_{S1}-casein. None of the samples showed heat-induced coagulation when heated at 116 °C. However, the results of the heat stability test showed that heat stability is reduced by addition of calcium in that pH range.

Relationship between milieu conditions and casein micelle size

Changes in casein micelle size were often correlated with the photometrically measurable turbidity changes of milk (van Boekel et al. 1989b, 1989a). The major problem of the insufficient specifity of turbidity measurements, especially when whey proteins are present, was often discussed as changes in size can be due to an increase in radii of micelles ('swelling') or dissociation of caseins.

Our hypothesis was then that heat-induced coagulation is preceded by an increase in particle size as observed in concentrated skim milk in all studies on that topic using particle sizing techniques (Dumpler and Kulozik 2016; Dumpler et al. 2017c; Dumpler et al. 2017b). We assumed that there is a close linkage between calcium activity and casein micelle size. In addition, it was uncertain if other milieu conditions are necessary to induce coagulation as addition of calcium chloride in the range investigated and a pH as low as pH 6.35 did not result in complete coagulation of MCC. We therefore investigated changes in particle size for all milieu conditions that we investigated in relation to casein dissociation.

Fig. 8-8 shows the results for the a particle size of casein micelles expressed as the volume based parameter $d_{50,3}$. The pH was in this case was 6.65 in the MCC diluted 1:20 by SMUF during PCS measurements. This means that all changes observed after heating must were irreversible as all samples had the same pH after dilution with SMUF. Fig. 8-8a shows that casein micelle increases in size over time at different heating temperatures. It can be seen that the rate of increase in size of casein micelles increases with increasing heating temperature. The end of the curve indicates the onset of gel formation at 124, 132, and 140 °C. This could be due to the increase in volume fraction of casein micelles that tends towards unity with increasing micelle size. Dissociation of caseins (Fig. 8-3a) was not directly related to changes in size (Fig. 8-8a) as the progress over time was found to be different. The observed gel formation could therefore be also linked to the increase in micelle size. However, changes in micelle size did not affect MCC viscosity to a large extent when no gel formation was observed. Therefore, aggregation due to structural changes might be more important than the theoretical volume fraction of casein micelles in MCC. At 108 and 116 °C, no increase in casein micelle size was observed in the time range investigated.

Fig. 8-8b shows the effect of the initial pH before heat treatment on casein micelle size over time. We found that compared to the initial $d_{50,3}$ of unheated casein micelles, the size slightly decreased at pH > 6.8 which could result from the dissociation of caseins from the micelles as also observed by Taterka and Castillo (2015) and Anema et al. (2004). A strong increase in casein micelle size and subsequent coagulation at pH 6.0 in SMUF was also observed by Mounsey and O'Kennedy (2009). This shows that a sufficiently low pH and a high soluble calcium level alone can also lead to coagulation, even without heat treatment.

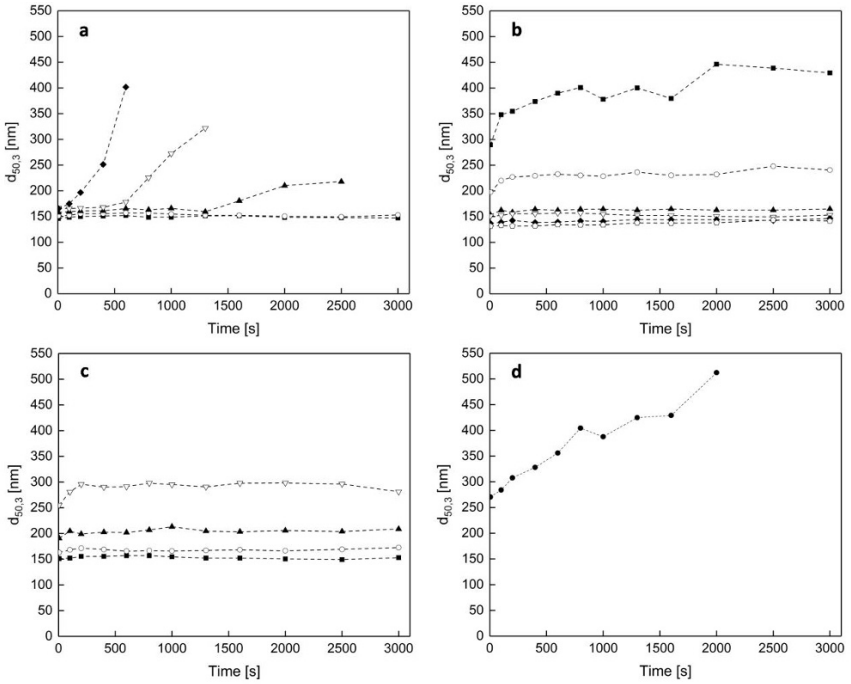

Fig. 8-8: Volume based average diameter $d_{50,3}$ of casein micelles in heat treated MCC at 108 °C (■), 116 °C (○), 124 °C (▲), 132 °C (▽), and 140 °C (◆) is shown in (a). The $d_{50,3}$ of casein micelles in MCC heat treated at 116 °C adjusted to pH 6.35 (■), 6.5 (○), 6.65 (▲), 6.8 (▽), 6.95 (◆), 7.1 (⬠) depending on heating time is shown in (b). Panel (c) shows the $d_{50,3}$ of MCC heat treated at 116 °C after addition of 0 (■), 1 (○), 2 (▲), and 3 mM (▽) soluble calcium as calcium chloride and panel (d) shows the $d_{50,3}$ of MCC depending on heating time at 116 °C after addition of 2 mM soluble calcium, 140 mM KCl, and pH adjusted to pH 6.35 (●).

Interestingly, when we consider that about 40% of overall casein was dissociated from casein micelles at pH 7.1, the decrease in average size of casein micelles is comparably small. At low pH and high calcium activity, the internal structure of casein micelles is weakened. However, casein monomers remain partly attached to casein micelles as could be observed at pH 6.5 and 6.35. This could be due to weakening of electrostatic bonds by adjustment of pH and hydrophobic bonds by heat treatment. We observed that the adjustment of MCC to a pH < 6.8 and subsequent heat treatment resulted in a relatively rapid increase in casein micelle size at the beginning and reaches a plateau over heating time.

The results of the addition of soluble calcium in Fig. 8-8c also indicate that changes in milieu conditions like pH and soluble calcium result in fast kinetic changes in casein micelle size when MCC is heat treated. A new equilibrium state in casein micelle hydration can be obtained without inducing coagulation of casein micelles.

Fig. 8-8d shows the results for a combination of milieu conditions likely to lead to coagulation. When the ionic strength was increased by addition of 140 mM KCl in addition to calcium chloride addition of 2 mM and subsequent adjustment of the pH to 6.35, the increase in particle size in MCC resulted in a steadier increase. This resulted in particle formation, i.e. severe coagulation, at heating times > 1600 s. Adjustment of MCC to pH 6.35 and addition of soluble calcium without increase in ionic strength resulted in fast gel formation at 116 °C (data not shown). Preliminary experiments heating MCC adjusted to pH 6.35 and addition of 140 mM KCl at 116 °C did not result in a marked increase in coagulum formation. Changes in particle size were similar to MCC adjusted to pH 6.35.

In addition to these measurements, we plotted the $d_{50,3}$ in the plateau over pH in Fig. 8-9 for MCC with added soluble calcium without the adjustment of pH and MCC with adjusted pH. This enables to differentiate between the effect of calcium on casein micelle hydration and the effect of pH. We therefore calculated the calcium activity at a certain pH from Fig. 8-6. Fig. 8-9 shows that at a higher pH, a higher calcium activity is necessary to obtain the same $d_{50,3}$. Hence, we can conclude that the decrease in pH does not only affect calcium activity and thereby affect casein micelle size. This means that the adjustment of pH as well as calcium addition individually and in combination affect casein micelle size.

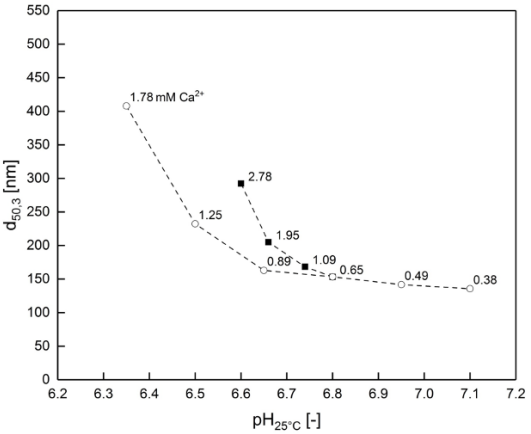

Fig. 8-9: The maximum $d_{50,3}$ of heat treated MCC depending on pH. The change in calcium activity was obtained by addition of calcium chloride (■) or adjustment of pH (○). Data captions indicate the determined calcium activity MCC at 25 °C.

Heat-induced coagulation of MCC depending on milieu conditions

As already mentioned, we could see that the addition of soluble calcium and the adjustment of MCC to pH 6.35 did not result in pronounced coagulation at 116 °C. An additional increase in ionic strength resulted in a complete coagulation of MCC. We therefore investigated the composition of the centrifugal (4,000*x*g/10 min; *closed symbols*) and ultracentrifugal supernatant (48,800*x*g/26 min; *open symbols*) in terms of individual caseins and also particle size. The results for dissociated casein in the ultracentrifugal supernatant were similar independent of milieu conditions. Samples without calcium addition and increase in ionic strength showed more dissociated κ-casein at shorter heating times. This indicates that a higher ionic strength and a higher concentration of soluble calcium increase the cohesive forces of caseins within the casein micelles as also seen in Fig. 8-7.

The relative amount of κ-casein and α_{S1}-casein in the supernatants in Fig. 8-10 shows that the addition of 3 mM Ca^{2+} alone did not induce coagulation. The adjustment of MCC to pH 6.35 led to a relatively rapid coagulation of about 20% of the casein without a further decrease. For MCC with 2 mM Ca^{2+}, 140 mM KCl, and pH 6.35, heat-induced coagulation set in at > 1600 s that led to complete coagulation of all caseins that had not dissociated. After 3000 s of heating at 116 °C, all casein micelles had coagulated in this MCC.

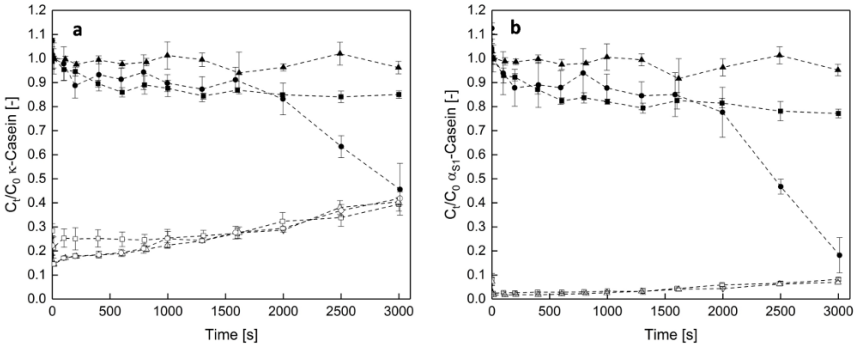

Fig. 8-10: Relative amount of dissociated κ-casein (a) and α_{S1}-casein (b) in MCC analysed in the ultracentrifugal supernatant centrifuged at 48,800*x*g/26 min (*open symbols*) and centrifugal supernatant centrifuged at 4,000*x*g/10 min (*closed symbols*) after heating at temperature of 116 °C, pH adjustment to 6.35 (■,□), and addition of 3 mM soluble calcium (▲,△). A combination of 2 mM soluble calcium, 140 mM KCl, and pH 6.35 is also shown (●,○).

Surprisingly, κ-casein coagulated together with calcium sensitive casein in whey protein-free MCC. This means that the dissociation of κ-casein is unrelated to heat stability of casein micelles. The increase in size possibly leads to exposure of calcium sensitive caseins and hydrophobic regions on the surface of casein micelles. Heat-induced dissociation of κ-casein was not pronounced at the onset of coagulation and is therefore not as strongly related to heat-induced coagulation as is the weakening of the

internal structure of the casein micelles. The formation of distinct particles was found when the ionic strength was increased in addition to low pH and calcium addition.

From these results, it is clear that calcium activity and pH are likely to be factors that define the equilibrium state of casein micelle hydration at a certain temperature. Gonzalez-Jordan et al. (2015) showed that a decrease in pH increase the mobility of phosphoserine residues in casein micelles. The heating temperature also represents a factor that can induce an ongoing change in casein micelle structure if the energy supplied is sufficient to break up hydrogen and hydrophobic bonds or electrostatic interactions between phosphoserines and CCP.

An increase in ionic strength, i.e. the addition of KCl in this case, competes with calcium for ionic bonds on the one hand, but fosters particle aggregation due to reduced steric and electrostatic repulsion of casein micelles on the other hand. The observed particle formation instead of gel might be preferred at high ionic strength and low pH due to the closer packing of casein micelles. Increased ionic strength could result in a lower degree of pH-, calcium-, and heat-induced destabilisation of casein micelles necessary to induce coagulation.

Particle size measurement of the resulting fractions were carried out to confirm that the result obtained by Dumpler and Kulozik (2016) and Dumpler et al. (2017c) were also valid for heat treated MCC and the observations made concerning heat-induced dissociation, formation of small submicellar particles, and coagulation of casein micelles to large particles were independent of the presence of whey proteins. We could see that when the casein micelles start to coagulate and become sedimentable, the dominant fraction in MCC is formed by small, non-aggregation submicellar particles in the size range of 10-80 nm (data not shown). The increased hydration and the formation of 'mini-micelles' and aggregates at high soluble calcium concentration of 100 mM even at room temperature were also observed by Müller-Buschbaum et al. (2007) using PCS.

8.4 Conclusions

In this study, we investigated the effects of serum composition on heat-induced dissociation, hydration and coagulation of casein micelles in micellar casein as a model system. Dissociation of κ-casein was not found to be immediately linked to heat-induced coagulation. The increase in temperature, modification of pH, calcium level, and additionally ionic strength was necessary to induce severe coagulation of casein micelles at high temperature. This led us to the conclusion that heat-induced destabilisation of casein micelles can take two different reaction routes. The weakening of the internal micellar structure by low pH, high temperature, and high calcium leads to the well-known visibly observable coagulum formation. This increase in hydrodynamic radius will consequently lead to an exposure of calcium sensitive and hydrophobic casein residues that lead to crosslinks between casein micelles. The packing density of the micelles, i.e. gel-like or particulate structures, the critical level of disso-

ciation, and the velocity of crosslink formation might be dependent on ionic strength. The dissociation pathway can also be related to heat-induced destabilisation of casein micelles supported by low calcium, high pH, and denaturable whey proteins. However, such products containing dissociated casein micelles might not cause immediate technological problems like poor mouthfeel or sediment formation during storage. A change in milieu conditions by mixing and further processing into products might reveal preceding destabilisation. This study points out that a new definition of heat stability of casein micelles may be necessary that refers to both, dissociated casein and coagulation of casein micelles, and defines the overall heat-induced damage on casein micelles depending on milieu conditions. By inversion of milieu conditions, e.g. pH, these changes are likely to be partly reversible. An overall dissociative milieu condition at high pH cannot cause coagulation under the same conditions as it physically moves proteins in the opposite direction.

Summary and contribution of the doctoral candidate

Milk powders and milk protein concentrates powders (MPC) are gaining importance in the food industry either for special dietary needs or as functional ingredients to create structure within foods as gels, foams or emulsions. These dairy powders are usually produced by a combination of membrane concentration and possibly fractionation of caseins and whey proteins by microfiltration, diafiltration, heat treatment and subsequent spray drying. The functionality of these powders is largely defined by the extent of the diafiltration process, the diafiltration medium used, and the subsequent heat treatment that can modify the structure of the casein micelles in MPC. Many factors affecting heat stability of milk, concentrated milk, and milk derivatives have been discussed in literature. However, a clear correlation between observable structural changes and the onset and propagation of heat-induced coagulation was still lacking.

This study was conducted to obtain insights into two aspects. Insights into structural changes of casein micelles in micellar casein concentrates during heat treatment depending on milieu conditions should be provided. From the scientific point of view, it was intended to gain further insights into the long standing problem of the reasons for heat-induced coagulation of casein micelles in concentrated milk using a simplified model system.

Using this micellar casein model system, it could be shown that heat-induced dissociation of κ-casein is not directly correlated with heat-induced coagulation of casein micelles. Contrary, the loosening of the internal structure of micelles at lower pH followed the trend of a decreasing heat stability of the micellar casein. Addition of soluble calcium and lowering the pH were found to be ancillary concerning the increase in casein micelle size upon heating as the change in pH affects both soluble calcium and the charge of caseins within the micelles. A combination of low pH, increased soluble calcium, and an increased ionic strength was required to induce co-

agulation of micellar casein under sterilisation conditions. However, both, dissociation of casein from the casein micelles as well as the weakening of the internal structure leading to coagulation should be denoted as heat-induced destabilisation of the colloidal stability casein micelles.

The major contributions of the doctoral candidate were the definition of the experimental set-up for diafiltration, spray drying of the micellar casein, reconstitution, and the heat stability tests. The continuous evaluation, plotting and calculation of data were performed by the doctoral candidate to provide the basis for the manuscript. The manuscript was primarily written by the doctoral candidate.

9 Overall discussion and outlook

Considerable attention has been attributed to the heat stability of concentrated milk in dairy research due to the hazard of coagulation under heating conditions unconcentrated milk is able to withstand. Critical temperature-time conditions of the heat treatment without coagulation, especially under continuous heating conditions and the total solids content of the concentrate were unknown. In addition, undesired heat-induced structural changes in concentrated milk eventually leading to colloidal destabilisation of casein micelles and the subsequent coagulation process have not yet been fully understood. The rate determining steps of this reaction leading to coagulation could not be identified, yet. The elucidation of the underlying mechanisms of the coagulation process is complicated mainly because of the compositional complexity of milk.

Natural variations in the multi-component milk system and the interaction of constituents in heated milk made it difficult, on the one hand, to induce targeted changes and to observe specific effects of heat, i.e. to find correlations between changes made and their respective effects. Mainly the milk salts, especially calcium, and the physico-chemical state of proteins were considered as relevant components affecting heat-induced coagulation of milk and concentrated milk (Sommer and Hart 1919; Singh et al. 1995; Crowley et al. 2014; Crowley et al. 2015). However, milk proteins as polyelectrolytes could also be considered as part of the milk salt system as they will be affected by changes in pH, ionic strength, temperature, divalent cations, addition of phosphates, and citrates that also affect each other (Walstra et al. 1984). On the other hand, methods to assess heat stability of milk were mostly based on the visual observation of a single point in the coagulation process and mostly at a fixed temperature. A targeted variation of the heating temperature was only performed by Davies and White (1966) for unconcentrated milk. A tracking of the coagulation process to derive kinetic parameters for unconcentrated milk was performed by White and Davies (1966) and White and Sweetsur (1977) to study the kinetics of the reaction. Nieuwenhuijse et al. (1991) followed the course of coagulation of concentrated milk at different pH values, but focused on the shape of the flocs rather than on kinetics. An attempt to study the kinetics of the coagulation of concentrated milk had not yet been carried out.

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7_9

9.1 Heat stability of concentrated milk

The subjective heat stability testing procedure at constant temperature as described in literature was used to some extent to study the temperature dependency of the heat coagulation time (HCT) by Davies and White (1966). From this approach, however, no information is obtained about the kinetics of heat-induced destabilisation of casein micelles, the reactions taking place, the course of the coagulation, and the extent of dissociation of casein from micelles. The objective test method can be regarded as superior with respect to the information obtained although it is much more time-consuming. This method was originally developed by White and Davies (1966) for unconcentrated milk to ascertain the validity of the subjective method. It could be shown that the visual determination of the onset of coagulation is relatively precise as heat-induced coagulation proceeds rapidly to form large protein aggregates after the initial *lag*-phase of complete stability, i.e. the HCT. However, it has to be born in mind that the coagulation time is an approximation to the actual sample stability due to the limited amount of coagulum to be formed for the visual detection of coagulation. A formal reaction kinetic model was proposed by White and Sweetsur (1977) for the coagulation process of unconcentrated milk when the initial *lag*-phase was neglected.

A very pronounced coagulation was also observed using the heat stability test as described in section 4.2, especially for concentrated milk. The higher the total solids content of the concentrate the more pronounced was the coagulum formation. In addition, slight variations in the temperature profile of the heat stability test markedly affected the coagulation time observed, especially when samples already coagulated during the come-up time to the final temperature. At first, the relationship between the coagulation temperature and time was uncertain. Therefore, these two parameters were recorded separately and taken both as a means to characterize the overall heat stability of the concentrates. Hence, a systematic approach to relate these two parameters was necessary.

The observation of the variation of the HCT with slight changes in the temperature-time profile, a lower heat stability of the concentrates with increasing total solids content, and results in literature suggested that the reactions in concentrated milk leading to coagulation can be described by kinetics. It was found that iso-effect lines for the visual coagulation of concentrated skim milk of different total solids content can be obtained as shown in Fig. 9-1 (Dumpler and Kulozik 2015). It is impossible to derive kinetic parameters by the visual determination of the coagulation point and the non-isothermal heat treatment in this batch heating system. Nevertheless, the relationship between the heat coagulation temperature and the corresponding coagulation times as a means to estimate the overall heat stability of CSM depending on its total solids content could be shown.

When we now consider the data obtained by the objective heat stability test of White and Davies (1966) and White and Sweetsur (1977), it is possible to state that the variable sample temperature, or better to say the variable rate constants, of the subjective heat stability test integrated over time until coagulation occurs gives a

measure for the overall heat stability, i.e. the overall heat load possible without coagulation. Straight lines obtained on a semi-logarithmic plot of *log*-coagulation time over temperature ascertained the hypothesis that the reactions leading to coagulation could be of kinetic nature and described by a reaction kinetic model. A constant sample temperature over time is therefore not a prerequisite to obtain comparable results as long as the heating profile defining the overall heat load is fixed. Actually, the heating temperature can be rather adjusted to the expected heat stability of the samples. This was done in literature by defining measurement temperatures of unconcentrated milk to 140 °C and 120 °C for evaporated milk at 18-22% total solids as unconcentrated milk might not coagulate at 120 °C at a reasonable timespan. The procedure in this study was extended to allow for the precise determination of the heat stability as a function of coagulation temperature and time of samples that show very low heat stability such as highly concentrated milk by adjusting the oil bath temperature in a wide range from 95-165 °C. For optimal precision and limited chemical changes, the heating conditions of the heat stability test should be chosen in a way that the coagulation times obtained vary between 200 and 1000 s.

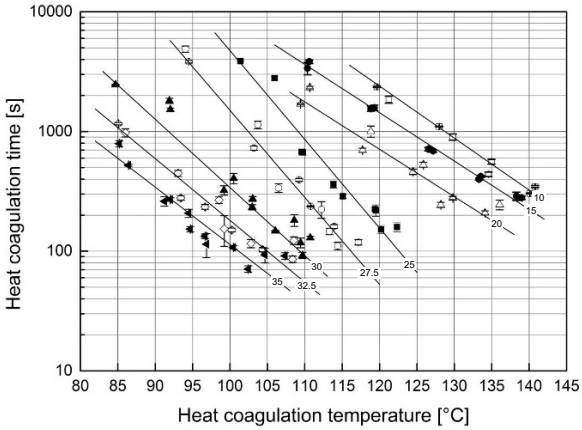

Fig. 9-1: Correlation between milk sample temperature and time of coagulation of concentrated skim milk of different total solids content. The total solids content is indicated on the regression lines (Dumpler and Kulozik 2015).

Variations in the visually detected HCT of different kinds of standard heat stability tests using the same batch of milk as performed by Kneifel et al. (1987) can therefore be attributed to variations in the sample temperature which was not recorded in most cases. On the one hand, the predefinition of the experimental setup and the measurement temperature by national standards was meaningful for industrial applications to ensure comparability of results. This was not given when unconcentrated milk and concentrated milk were compared using the HCT at 120 and 140 °C, respectively, as two parameters influencing HCT were varied. For research purposes, a

variation of measurement conditions, especially concerning the objective test method, might have cleared the way for more studies on kinetics of coagulation and dissociation of proteins in various milks and concentrated milk, and factors affecting these two opposite reactions in the past decades. Especially in combination with studies on the coagulation process, far more information on the heat stability of a sample can be achieved. This information is seen as a prerequisite for fundamental mechanistic insights. In order to define critical levels of aggregated protein, a description of the temperature dependency of the HCT depending on total solids content of the concentrate in the case of RO concentrated skim milk or milieu conditions is required. Therefore, isothermal heat treatments both on pilot scale and on lab scale were performed for the estimation of kinetic parameters of the heat-induced aggregation of concentrated skim milk. Iso-effect lines could be derived from the latter heat treatment trials taking a critical level of 2% of sedimentable protein as an indication for the onset of coagulation as shown in Fig. 9-2.

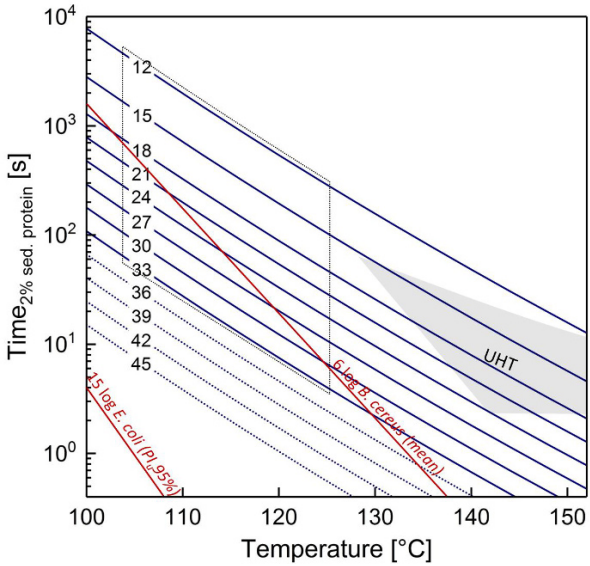

Fig. 9-2: Iso-effect lines for CSM for a critical level of 2% of sedimentable protein of total protein depending on heating temperature, heating time, and total solids content of bulk CSM (section 7.3)

A comparison between the iso-effect lines in Fig. 9-2 and the critical temperature-time combinations obtained on pilot scale using DSI (section 4.3) showed that holding times > 6s could be modelled by using the kinetic parameters of section 7.3 to calculate the amount of sedimentable protein as will be explained in section 10.1. In all cases, the relationship between the overall heat load expressed as temperature-time combinations and total solids content of the CSM was found to be valid using three

different heating systems. The denaturation of whey proteins was not found to interfere with the coagulation process by crosslinking of casein micelles to a large extent when heated quickly at >100 °C.

The similarities between Fig. 9-1 and Fig. 9-2, explained by the kinetic nature of reactions leading to coagulation, makes it possible to estimate the heat stability of milk systems, e.g. milk fat containing concentrated milk, MF retentate, UF retentate, infant formula, by the subjective heat stability test on lab scale. The prerequisite is that the heat stability of the sample is within the heat stability range investigated and that the temperature dependency of the coagulation process is similar to that of CSM. The heat coagulation temperature and heat coagulation time of the lab scale test can then be taken to find a corresponding dry matter of concentrated skim milk in Fig. 9-1 that shows approximately the same heat stability. For this corresponding total solids content, the line of equal effect in Fig. 9-2 is then used to determine the maximum stability time at the constant target heating temperature for continuous heat treatment. If non-isothermal continuous indirect heat treatment on pilot and industrial scale will be applied, the temperature time-profile needs to be integrated over heating time to calculate A* that indicates if the sample will withstand the required heat treatment as described in section 10.3. The feasibility of the transfer of the heat stability test results to other casein containing systems and the prediction of maximum temperature-time combination using Fig. 9-1 and Fig. 9-2 could be proven by the application of these considerations to milk fat containing concentrated milk on industrial scale direct steam injection and infusion heat treatment as will be discussed in section 9.3.

9.2 Mechanisms of the heat coagulation of casein micelles

Many hypotheses have been formulated on the reactions involved in heat-induced coagulation. Attempts were made to characterize the decreasing colloidal stability of casein micelles during heating based on particle-particle interaction forces to characterize the stability of casein micelles (Rose 1962; Singh and Fox 1987c; van Boekel et al. 1989a; Nieuwenhuijse et al. 1991; O'Connell and Fox 2000). However, the nature and extent of the change on the casein micelle surface and milieu conditions to allow for a lasting contact between casein micelles after collision induced by Brownian motion to overcome the residual energy barrier remained uncertain. The effects of milieu conditions that will affect both the destabilisation of casein micelles and the kinetics of aggregation have been understood in terms of their effect on HCT. Numerous investigations were conducted to relate heat-induced coagulation with the dissociation of κ-casein from casein micelles in milk and concentrated milk. This was investigated depending on various milieu conditions and the interaction and dissociation in the presence of whey proteins (Singh and Fox 1985a, 1985a, 1986, 1987c; Anema and Klostermeyer 1997; Anema 1998; Anema and Li 2000; Anema 2008). However, a correlation between the dissociation of κ-casein and the onset of heat-

induced coagulation over the entire pH range relevant for heat stability testing (pH 6.2-7.3) could not be established. Therefore, other chemical and physical reactions must be involved that lead to the colloidal destabilisation of casein micelles, especially at pH < 6.7 to induce coagulation.

A weakening of the internal structure of the casein micelles as proposed and discussed in section 8.3 had not yet been considered in literature. Dissociative conditions at pH > 6.7 for caseins, especially κ-casein and a coincident low calcium activity appeared to be opposite to conditions that cause coagulation, i.e. high calcium, low pH, and high ionic strength. So we could state that dissociation of caseins, especially κ-casein is not immediately linked to coagulation of the micelles. Nevertheless, internal structural changes and dissociation of caseins induced by heat under these two conditions, i.e. pH > 6.7 or pH < 6.7, have to be seen as a noticeable change of the native state of casein micelles which is dependent on both the deviation from the natural milieu conditions and the severity of the heat treatment. A deviation from natural conditions causes either heat-induced dissociation or internal structural disintegration that subsequently causes coagulation. The increase in size of the casein micelles due to a weakening of the internal structure of the casein micelles in micellar casein at pH < 6.5 was observed in all heat treated CSM samples at their natural pH independent of the heating method. The increase in size was accelerated by increased total solids content and coincident also a lower pH of CSM and higher heating temperatures (section 5.3) which parallels the observations simulated in section 8.3 using micellar casein. These conditions were found to be required to induce coagulation.

In the first step of heat-induced coagulation of casein micelles, we might have to differentiate between factors contributing to a loosening of the internal structure of micelles at low pH accompanied by limited dissociation of caseins under heat and the dissociation of caseins without an increase in micelle size. Casein micelle size increases by a heat treatment depending on heating temperature and time, whereby a low pH and high soluble calcium concentration increase the rate of this process. In the case of unconcentrated milk, the comparably small increase in size was attributed to the denatured whey proteins that become attached to the casein micelle surface (Anema and Li 2003a). In micellar casein, in the absence of whey proteins, the remarkable increase in hydrodynamic radius must be a result of a weakening of bonds within the casein micelles. It was found that an increase in soluble calcium by addition of calcium chloride or a decrease in pH (pH < 6.7) resulted in a decrease in dissociated caseins from casein micelles. This indicates that electrostatic bonds induced by the presence of calcium, i.e. calcium bridges, inhibit the complete dissociation of caseins. Contrary to this, the dissociation of casein from the micelles occurs at high pH under heat. At pH > 6.7, the caseins, especially κ-casein readily dissociate when whey protein free milk is heated without immediate coagulation. Due to the low calcium activity and high surface charge of the casein micelles, the energy supplied by heat is sufficient to cause complete dissociation of caseins. In both cases, heat supplies the energy for a rearrangement of the casein micelles whereby the final structure of the casein micelles after heating is dependent on milieu conditions, mainly pH and calci-

um that affect electrostatic interactions of the caseins within the micelles. We could categorise pH > 6.7 as dissociating conditions that do not cause coagulation at low ionic strength. However, at high ionic strength, pH > 6.7, and in the presence of denaturable whey proteins, the heat stability of concentrated milk was found to be low due to aggregation of κ-casein depleted micelles under these conditions (Singh and Fox 1987c; Nieuwenhuijse et al. 1991; McCrae and Muir 1995; Dumpler and Kulozik 2015).

Destabilisation of micelles by enzymatic hydrolysis of κ-casein was found to destabilise milk at all pH values whereby excessive renneting was required to induce a marked destabilisation of casein micelles (Fox and Hearn 1978c). This indicates that the removal of some κ-casein to an extent as observed in the studies on concentrated skim milk and micellar casein does not destabilise casein micelle sufficiently to induce coagulation. In contrast to this, chemical or enzymatic crosslinking of caseins stabilised casein micelles against heat-induced coagulation over the entire pH range. Singh and Fox (1985b) and Nieuwenhuijse et al. (1991) found that the internal crosslinking of casein micelles by formaldehyde increased the heat coagulation time over the entire pH-range (pH 6.4-7.3) with increasing concentration of formaldehyde in milk and concentrated milk, respectively. This was explained by these authors by the reduced dissociation of κ-casein. As the dissociation of κ-casein is relevant at pH > 6.7, this would not explain the stabilizing effect of crosslinking at low pH (pH < 6.5) with increasing concentration of formaldehyde. When we now integrate the results presented in section 8.3 on the weakening of the internal structure of the micelles that causes an increase in the calcium sensitive surface of micelles, we could assume that covalent crosslinking of caseins within the micelle as described by Bulca et al. (2016) reduces the rate of the increase in casein micelle size and thereby the exposure of calcium sensitive caseins on the surface of the micelles. Thereby, covalent crosslinks reduce the tendency of casein micelles to aggregate at low pH.

In the current theory, 'salt-induced' aggregation due to low surface charge and high ionic strength was given as an explanation for the low heat stability of concentrated milk at low pH (Nieuwenhuijse et al. 1991). This is, however, not a continuous change of micelles or the serum induced by heat preceding coagulation and would result in immediate coagulation of casein micelle if appropriate as a single reason. No changes in structure of micelles except for the coverage of micelles with whey proteins that inhibit aggregation to some extent had been investigated (Singh and Fox 1987c).

A reduced rate of internal disintegration of casein micelles due to covalent thermal crosslinking and the association of whey proteins on the micelle surface could also be two of the factors that explain the effect of preheating of milk to increase heat stability of concentrated milk at pH < 6.7. However, the stabilising effect was less pronounced at low pH as less structural changes might be necessary to induce coagulation in the presence of high soluble calcium concentrations in concentrated milk of high total solids. A multiple involvement of calcium in the coagulation process could be a reason. A weakening of the internal structure that became detectable as an in-

crease in hydrodynamic radius at pH < 6.5 could not be detected at higher pH and is therefore correlated with the increase in heat stability of micellar casein.

In general, predictions about the bonds affected within the casein micelles by changes in milieu conditions and temperature are complicated by the unknown internal structure of casein micelles. Hypotheses can be formulated based in the nature of the components added and the proposed structural models of the casein micelles which suggest that calcium bridges and ionic bonds will be affected by changes in pH.

At pH > 6.7, the rate of dissociation of casein from micelles increases. Denatured whey proteins attached to casein micelles via κ-casein by thiol disulphide exchange will become increasingly detached from casein micelles at pH > 6.7 with increasing heating intensity (Singh and Fox 1987c; Anema and Li 2000). However, the increased surface charge might prevent heat-induced coagulation together with a low calcium activity. However, the second step of heat-induced aggregation of destabilised casein micelles is hampered due to low calcium activity and high surface charge of the micelles. A high surface charge of micelles and a reduced calcium solubility which results in a reduction of the attractive forces between κ-casein depleted casein micelles result in an energetic barrier towards coagulation that needs to be overcome by heat-induced collision of casein micelles (Singh 2004). In addition, calcium might also induce the crosslinking of casein micelles during heat-induced aggregation. This means that soluble calcium plays a crucial role in both, the stabilisation of the internal structure of casein micelles and the coagulation of casein micelles as it increases the hydration of casein micelles and might also take part in calcium bridges to form coagulated casein micelles.

Therefore, the choice of the Weibullian model appears meaningful as a first attempt to model this reaction instead of using a multi-step kinetic model. Not all factors affecting the colloidal stability of the casein micelle and their individual kinetics might be known to date. The length of the *lag*-phase, characterized by colloidal stability and the subsequent coagulation process of casein micelles in concentrated skim milk (pH < 6.7), could be described by a Weibullian model which suggested that the preceding destabilising reaction proceeds and leads to an increased rate of coagulation of casein micelles over time (section 7.3). An Arrhenius relation for the temperature dependency of the coagulation reaction in the temperature range investigated could be established. The results of Nieuwenhuijse et al. (1991) suggested that, although the shape of the coagulum was very different depending on the initial pH, the modelling of the coagulation process by the Weibullian model is likely to be appropriate also for different pH values at constant total solids and heating temperature over time. The amount of coagulable protein was approximately constant independent of pH. In some studies, a multi-step coagulation process was observed (White and Davies 1966; Muir and Sweetsur 1978; O'Connell and Fox 2000). This observation could be attributed to a non-quantitative separation of aggregates by low centrifugal forces applied that resulted in incomplete sedimentation of aggregates (Nieuwenhuijse et al. 1991).

The second step of the coagulation process is of a kinetic nature driven by heating temperature and time. We suggest that milieu conditions, mainly the initial pH, additionally heat-induced changes in pH, calcium activity, and chemical degradation reactions upon prolonged heating will decide if strong coagulation or poor coagulation is observed. High ionic strength, low pH, and high ionic calcium level will induce strong coagulation due to the observed weakening of the internal structure of the casein micelles and increase in the calcium sensitive surface, crosslinking by calcium, and salting out effects. It can be stated that both the dissociation of casein and the weakening of the internal structure of casein micelles result in a disruption of the native micellar structure. The aggregation of micelles will lead to visible coagulum formation and the dissociation of caseins will lead to soluble casein that is not noticeable as instability by the visual observation of the sample. Both reactions might be present in a heated sample, whereby the initial pH, the presence of whey proteins, the amount of soluble calcium, heat-induced acidification, and other compositional aspects will decide which of these two reaction paths dominates. Therefore, the subjective test will only give an indication of heat-induced changes when coagulation is the dominant reaction path as it is the case for concentrated milk at its natural pH. The determination of both dissociated and aggregated caseins and whey proteins will be relevant to determine the overall heat-induced changes under certain milieu and heating conditions.

9.3 Changes in heat stability induced by preheating, milk fat, and homogenisation

Preheat treatment

The improvement of the heat stability of concentrated milk by preheat treatment of milk before concentration, especially evaporated milk in the total solids range of 18-22% total solids, has been investigated since the 1930s (Deysher et al. 1929; Webb and Holm 1932; Miller and Sommer 1940). Most investigations concerning the preheat treatment of milk for evaporated milk manufacture focused on a fixed total solids content and a variation in the initial pH prior to heat stability testing. The results showed that the effect of preheat treatment of milk can be attributed to a shift in the HCT-pH profile to the acid side, i.e. closer to the natural pH of concentrated milk (pH < 6.6). Preheating of unconcentrated milk and subsequent heat stability testing results in a reduction in the observed HCT. This observation was attributed to a shift in pH away from the pH of maximum stability of unconcentrated milk as well as heat-induced damage of casein micelles by dissociation of κ-casein (Walstra et al. 1984; Singh and Fox 1986). In addition, preheat treatment of milk reduces the reactivity of whey proteins in milk. The heat-induced interaction of κ-casein with whey proteins on the casein micelle surface in unconcentrated milk also forms a steric barrier of denatured whey proteins against heat-induced aggregation of the subsequently concentrated milk at pH < 6.7 (Fox and Hearn 1978a). Covalent crosslinks within the casein micelles induced by degradation products could also play a role to stabi-

lise casein micelles at pH < 6.5. These crosslinks are formed by chemical reactions of amino acid side chains with aldehydes formed from lactose and β-elimination in amino acid side chains and subsequent crosslink formation. Covalent crosslinks reduce the dissociation of caseins or a loosening of the internal structure of the casein micelles.
Changes in the mineral equilibrium of milk, mainly the precipitation of calcium phosphate that reduces the soluble serum calcium upon prolonged heating have also been discussed (Nieuwenhuijse et al. 1988).
A considerable increase of the heat coagulation time could be achieved by preheating conditions that denature a large proportion of the whey proteins. However, the amount of denatured whey proteins necessary to induce heat stability of the concentrates and nature of the whey protein aggregates adsorbed to the casein micelle surface is uncertain whereby the second transition to complete unfolding of β-lg at temperatures > 140 °C could play an important role (Walstra et al. 1984). Recommended preheating conditions vary widely from 90 °C for several minutes to UHT conditions (Muir 1984). Optimal conditions were found to vary also within season (Singh and Tokley 1990). In addition, little is known about the heat stability of concentrated milk > 22% non-fat total solids and the effectiveness of preheat treatment of milk on concentrated milk of higher total solids content. If heating temperatures < 100 °C for storage of highly concentrated milk before spray drying are considered as heat treatment, it was found that the structural changes in the concentrates rather resemble gel formation than heat-induced aggregation and particle formation. Trinh et al. (2007) investigated the onset of the increase in viscosity as indicator for coagulation in reconstituted whole milk concentrates from medium heat powder. They found the time to onset of the increase in viscosity decreasing with increasing heating temperature and decreasing total solids content in the range 40-48% total solids at 65 °C and heating temperatures of 55-85 °C at 46% total solids. Again, a relation between total solids content of the concentrate, heating temperature and the timespan required to induce structural changes was found. Webb et al. (1943) described the effect of preheating on concentrated whole milk of various total solids contents up to 34% total solids (≈ 29.5% non-fat total solids) for batch sterilisation. They found that preheating of milk in the UHT region was most effective to increase the heat stability of the concentrate.

In this study, a quantitative description of the increase in heat stability by preheat treatment of skim milk expressed as critical temperature-time combinations was described in section 4.3 for continuous heat treatment by direct steam injection. However, a rapid screening of the effect of different preheating conditions depending on total solids content was only possible by lab scale experiments as shown in Fig. 9-3 for a few selected heat treatments.

Preheating conditions of milk were varied in a wide range for this screening. Both parameters, coagulation temperature and coagulation time over total solids of tested preheating conditions over CSM total solids are shown. The advantage of the developed heat stability test, measuring both heat coagulation temperature and time, as

compared to the standard test is that concentrated milk of various total solids can be characterized for its heat stability. Coagulation during the come-up phase is recorded by both the coagulation temperature and time.

It can be seen from the graphs that, in general, the heat stability of the non-preheated concentrated skim milk is decreasing with increasing total solids. Indirect preheat treatment of milk including a holding time of 60-80 s in range of 90-95 °C and a subsequent heating-up to the UHT temperature resulted in a decrease in heat stability of CSM < 15% total solids depending on the heating intensity. At higher total solids content, the heat stability of CSM was increased depending on the preheat treatment applied. Heating milk at 90 °C for 80 s resulted in no noticeable change in heat stability independent of the total solids content of the concentrate. The additional heating at 135 or 142 °C resulted in a noticeable increase in heat stability of the concentrate in terms of heat coagulation temperature and time. Heating milk to UHT conditions for a few seconds without a pre-holding time at 90 °C did not markedly increase the heat stability of concentrates > 20% total solids (data not shown). The irregular pattern of increased and decreased heat stability of preheated CSM indicates multiple complex reactions affection heat stability as discussed previously.

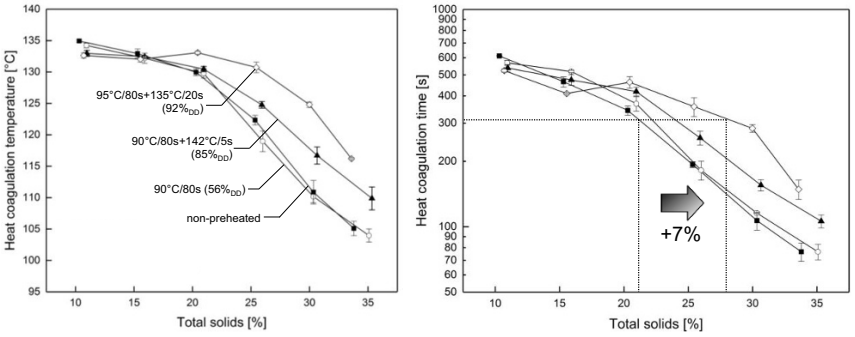

Fig. 9-3: Visually determined heat coagulation temperature (*left*) and heat coagulation time (*right*) of concentrated skim milk on lab scale depending on the total solids content. The preheat treatment conditions of unconcentrated milk before concentration by RO are indicated. The resulting degree of denaturation (DD) is also indicated in brackets.

The increase in heat stability can also be expressed as the increase in the total solids content that shows the same heat stability due to the close relationship between heat coagulation temperature, time, and total solids content of the concentrate. In order to obtain critical temperatures and times on lab scale, a concentrate from unheated milk is compared to that one from preheated milk (Fig. 9-3, *right*). The critical temperature-time combination of concentrate of 28% total solids from preheated milk heated at 95°C/80 s + 135 °C/20 s showed the same heat stability as non-preheated concentrate of 21% total solids. Despite the higher degree of denaturation of this UHT heat treatment using a longer holding time at 135 °C compared to 90°C/60 s + 142 °C/5 s, whey protein denaturation itself was not directly correlated to the increase in heat

stability. Therefore, denaturation and complete unfolding of β-lg at temperatures > 140 °C at the casein micelle surface might at least be partly responsible for the increase in heat stability. Using bulk milk from southern Bavaria, indirect UHT preheat treatments or alternatively, high heat treatments in the range of 115-125 °C/< 2 min were most effective to increase heat stability. The specific line of equal effect in Fig. 9-2 corresponding to the non-preheated total solids content can then be used for a quantitative estimation of the temperature-time combinations possible for continuous high heat treatment of CSM.

Using the standard heat stability test for evaporated milk as described in literature, the procedure was essentially the same. In order to judge if a sample of concentrated milk will coagulate during heat treatment, the length of the HCT as determined on lab scale and the sterilisation times of the industrial autoclave needed to be compared. Samples that coagulate during heating could not be estimated for their allowed T-t combinations without coagulation using the standard methods described in literature.

Milk fat and homogenisation

The comparison procedure of the lab scale experiments (Fig. 9-1) and the lines of critical temperature-time combinations for continuous heat treatment in Fig. 9-2 are useful to estimate the heat stability of concentrated milk containing different proportions of milk fat. It can be seen from the graphs in Fig. 9-4 that an increasing proportion of milk fat at the same overall total solids content results in an increase in the heat coagulation temperature and heat coagulation time.

The proportion of milk fat added as unhomogenised cream after concentration of skim milk by RO is indicated as labels. CSM (max. 0.1% fat) is shown for comparison. The increase in heat stability at the same total solids level with increasing fat content suggests that milk fat has no effect on heat stability of concentrated milk.

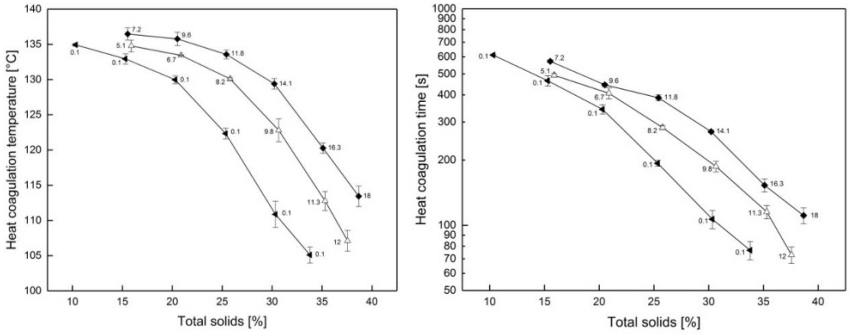

Fig. 9-4: Visually determined heat coagulation temperature *(left)* and heat coagulation time *(right)* of concentrated milk containing various proportions of milk fat depending on the total solids content. Milk fat was added as unhomogenised cream. Data labels represent the relative proportion of milk fat based on total volume of concentrated milk.

Huppertz (2016) summarised the results reported in literature showing that non-homogenised cream had little effect on the heat stability of concentrated milk up to 20% non-fat total solids. However, it was assumed that the volume exclusion effect of milk fat increases the total non-fat solids concentration in the water phase of concentrated milk. The more milk fat present in a certain volume of concentrated milk, the higher the concentration of non-fat components in the water soluble phase of concentrated milk at the same non-fat total solids content. This might become especially relevant for full cream concentrated milk at high total solids content and might become significant in terms of residual heat stability of the concentrate. To account for this effect, we calculated the volume corrected non-fat total solids content $TS_{non\text{-}fat,\ vc}$ by correcting the non-fat total solids content $TS_{non\text{-}fat}$ by the volume fraction of fat Φ_{fat} and the respective density ratio ϱ_{CSM} of CSM and milk fat globules ϱ_{fat} as shown in eq. 9.1. The density of the milk fat globules was assumed constant as 0.94 kg m^{-3} at 100 °C. The density of CSM was calculated from regression analysis of density over total solids content as determined by bending vibration.

$$TS_{non\text{-}fat,\ vc} = TS_{non\text{-}fat} \cdot \frac{1}{1 - \Phi_{fat}} \cdot \frac{\varrho_{CSM}}{\varrho_{fat}} \tag{9.1}$$

If no effect of the addition of unhomogenised cream would be observable, when the volume exclusion effect is considered, then the heat coagulation temperature and heat coagulation time should be equal at the same volume corrected non-fat total solids content. This should be true independent of the fat content of the concentrated milk. The results are shown in Fig. 9-5. It shows that the correction of the total solids content in the water phase can be used to estimate the heat stability of the milk fat containing concentrated milk.

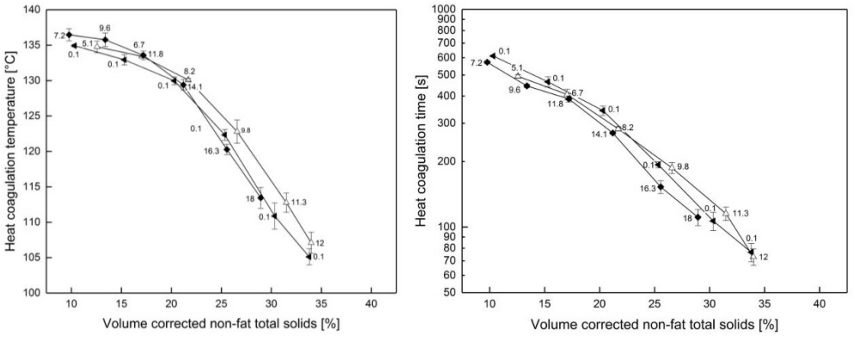

Fig. 9-5: Visually determined heat coagulation temperature *(left)* and heat coagulation time *(right)* of concentrated milk containing various proportions of milk fat depending on the volume corrected non-fat total solids. Data labels represent the relative proportion of milk fat based on the total volume of concentrated milk.

To reduce the rate of creaming of the milk fat during storage, homogenisation is usually applied as described in section 1.4.2. Homogenisation of the milk or the concen-

trate before or after preheat treatment or after concentration before sterilisation markedly reduces the heat stability of the concentrate with increasing homogenisation pressure, i.e. decreasing fat globule size, and increasing fat content (Sweetsur and Muir 1982, 1983a; Muir 1984; Hinrichs et al. 1998). In the case of continuous heat treatment of the concentrate, homogenisation can be performed aseptically after sterilisation. This option offers the opportunity that the heat stability of the concentrate is unaffected and can be estimated from the non-fat total solids content and the volume fraction of milk fat as described. In the case of homogenised milk fat globules, the secondary fat globule membrane makes the milk fat globules take part in the coagulation process and integrate into the coagulum which partly explains the reduced heat stability as the apparent volume fraction of the proteins increases (McCrae and Muir 1995; Huppertz 2016).

Homogenisation of the concentrate compared to unconcentrated milk at the same pressure level causes a higher homogenisation effect due to the higher viscosity of the continuous phase in the concentrates. However, homogenisation of the concentrate causing the same homogenisation effect causes a more pronounced reduction of the heat stability. Sweetsur and Muir (1982) and Sweetsur and Muir (1983a) found that the HCT of the concentrates were reduced to about 10% when concentrates were homogenised at > 200 bar, whereby the homogenisation of whole milk under these conditions reduced the HCT of the concentrates to about 20%. Hinrichs et al. (1998) found that the residual heat coagulation time was about 40% when milk was homogenised at > 200 bar before concentration by nanofiltration. If homogenisation is applied before heat treatment of the concentrates, the reduction in heat coagulation times (and the heat coagulation temperatures) could also be used to estimate the residual heat stability on industrial scale using the described heat stability test in combination with Fig. 9-1 and Fig. 9-2. In milk concentrates containing milk fat, the investigation of heat-induced aggregation would have been impossible, especially when homogenisation would have been applied. This would have resulted in complications in terms of sample preparation and analysis. Especially the determination of the particle size distribution of heat-induced casein aggregates would not have been possible due to an overlap of the size of casein aggregates and milk fat globules. Therefore, it was meaningful to increase the complexity of the system by integration of milk fat at a later stage and to transfer the results obtained for CSM.

9.4 Conclusion and outlook

In principle, heat stability of milk and concentrated milk was defined as the stability to withstand high heating temperatures. This might explain the 'choice' of the relevant pH-range from pH 6.3-7.3 usually investigated in literature. The heat stability of milk heated at 140 °C and concentrated milk of 20% total solids heated at 120 °C is sufficient to give meaningful differences and results within this range in most cases. Lower pH values result in the coagulation during heating or even coagulation at

room temperature, which is known as acid gelation. Higher pH-values (pH > 6.7) will result in pronounced (reversible) dissociation of casein micelles and no coagulation will be observable in the case of unconcentrated milk. Strong coagulation will be observed in concentrated milk, whereby at alkaline pH values, e.g. pH > 8 during alkaline cleaning in place, casein micelles are dissociated even in concentrated milk. Therefore, it can be stated that the pH range investigated, where an additional high heat treatment of concentrated milk is possible without immediate coagulation or dissociation, is narrow compared to the absolute pH range.

The intensity of the heat treatment that is applicable as defined by heating temperature and time depends on the tendency of the casein micelles to undergo a conversion into another state under certain milieu conditions. The (irreversible) conversion rate, i.e. the kinetics of the reaction from native state to equilibrium will be affected by the potential between the original state and the new state that is characterized by changes in milieu conditions such as pH, ionic strength, calcium, and others. Possible states of caseins micelles can be the coagulation of casein micelles to form gels, particulate structures, and the formation of dissociated monomeric casein or dissociated casein in the form of small colloidal particles. The relative proportions of these fractions will largely depend on milieu conditions and heating intensity. It can be concluded that heat-induced changes on casein micelles manifest themselves in at least three phenomena, i.e. heat-induced dissociation of caseins, an increase in casein micelle hydrodynamic radius, and the aggregation of casein micelles. Thereby, the kinetics and the reaction route is determined by milieu conditions. All these states must be denoted as *non-native*. The additional aspect of the weakening of the internal structure of casein micelles, mainly by heating temperature-time relationships, low pH, and high serum calcium making casein micelles prone to aggregation had not been considered in literature in relation to heat-induced coagulation. The internal dissociation of casein micelles, detectable by an increase in the hydrodynamic radius, drastically increases the surface area of casein micelles that is not shielded by κ-casein (section 8.3). Research on the bonds affected by changes in the environmental conditions is required for further insights into casein micelle stability. Coagulation is only a single peculiarity of (visible) instability of casein micelles. A deviation from the natural environment will necessarily create a tendency of casein micelles to undergo a conversion from the native state into dissociated caseins, gels, or particulate structures, especially when heat treated. Non-covalent bonds will increase or decrease in strength and the energy minimum of the protein structure will change. The velocity of the conversion is determined by factors influencing the kinetics of the equilibration of the system.

Therefore, industrial processes such as concentration, diafiltration, and heat treatment as well as milk concentrates produced need to be further investigated in relation to product stability, processing equipment and parameters. This is especially relevant when the liquid concentrates need to exhibit excellent storage stability.

10 Supplementary material

10.1 Supplementary note I

The modelling of sediment formation during direct steam injection heat treatment of CSM using short holding times at ultra-high temperature as investigated in section 4.3 and modelled by using data obtained in section 7.3 is shown in Fig 10-1.

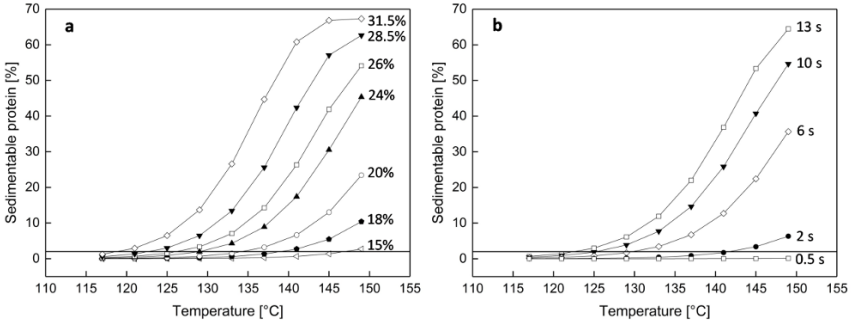

Fig. 10-1: Calculation of the expected relative amount of sedimentable protein based on the kinetic data of section 7.3 for direct steam injection heat treatments performed and investigated by Dumpler and Kulozik (2016) plotted over heating temperature and total solids (a) at a holding time of 9.5 s and depending on holding time at 27% CSM total solids.

A comparison of the onset of coagulation using the Weibullian model and the experimental data of section 4.3 is shown in Tab. 10-1. It was found that at longer holding times of around >6 s, it was possible to predict the amounts of sedimentable protein as well as the onset of heat-induced coagulation with sufficient precision. Nevertheless, it had to be taken into account that direct steam injection resulted in a higher amount of overall sedimentable protein as very little α_{S1}-casein and β-casein dissociated from the casein micelles as observed by Dumpler et al. (2017c) compared to indirect heat treatment in tubes as described in section 7.3.

Holding times of 2 and 0.5 s could not be modelled with sufficient precision in terms of sedimentable protein and the onset of coagulation. The reason for this observation might be that at very short holding time, there is a significant impact of

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https://doi.org/10.1007/978-3-658-19696-7_10

residence time distribution, possible fluctuations of the flow after steam injection, an increasing importance of the heating up ramp, and possible two phase flow of CSM and steam. These factors make it difficult to trust the calculations of mean residence time (RT) and its distribution. Although the mean residence time can be calculated easily based on tube volumes, the influence of these factors will become increasingly important with decreasing holding time, especially on pilot scale using small geometries. A broader RT distribution could explain the earlier onset of coagulation, the non-linear behaviour of CSM from preheated skim milk and lager amounts of sediment formed than expected by modelling. In addition, the datasets were extrapolated quite far to obtain the values described, especially for these short holding times. Modelling and comparison with experiment is instructive and can show technical as well as modelling limitations of holding times less than 1 s as an isothermal static indirect heat treatment is impossible in this range of temperature and holding times > 140 °C/< 2 s.

Tab. 10–1: Comparison of the observed and calculated temperatures of the onset of coagulation in CSM as investigated by Dumpler and Kulozik (2016) using direct steam injection heat treatment and derived from the Weibullian model used in section 7.3 using 2% sedimentable protein of total protein as a critical level.

Onset of coagulation [°C]					
Total solids [%][a]	Gompertz[b]	Weibullian model[c]	Holding time [s][d]	Gompertz[b]	Weibullian model
15	-	147	0.5	136.6	-
18	143	139.5	2	130.7	142
20	139.1	134	6	126.7	130
24	134.1	128	10	124.3	125
26	129.3	126	13	123.4	122.5
28.5	124.4	123			
31.5	120.6	118			

[a]Holding time was 9.5 s
[b]Measured and interpolated by the Gompertz-Fit in Dumpler and Kulozik (2016).
[c]Calculated according to the Weibullian model and results of section 7.3.
[d]CSM total solids content was 27%

10.2 Supplementary note II

Iso-effect lines for critical levels of sedimentable protein other than 2% as modelled in section 7.3 are shown in Fig. 10-2. These iso-effect lines can be used to estimate the possible temperature-time combinations for heat treatments of CSM having certain total solids content if a higher relative amount of aggregated protein than 2% is tolerable. Critical temperature-time combinations were calculated for 5% and 10% of sedimentable protein in Fig. 10-1 a and b, respectively. It can be seen that the complete

inactivation of vegetative microbial cells is possible for all concentrate total solids contents plotted without sediment formation also for highly concentrated milk.

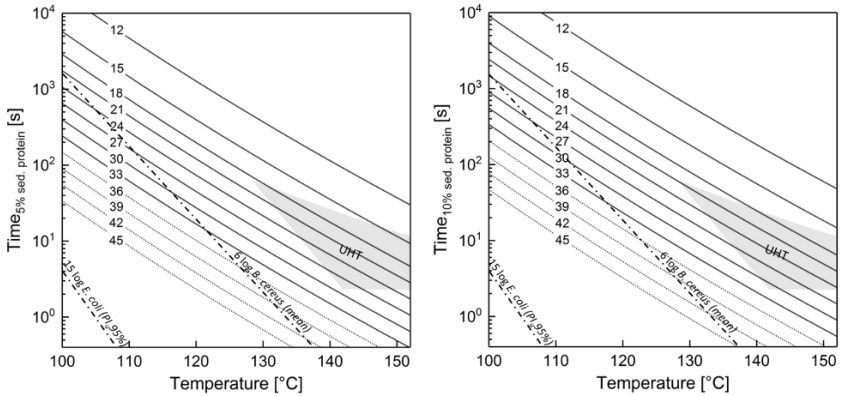

Fig. 10-2: Iso-effect lines for CSM for a critical level of 5% (*left*) and 10% (*right*) of sedimentable protein of total protein depending on heating temperature, heating time, and total solids content of bulk CSM. Extrapolated total solids contents (*dotted lines*) are shown, the UHT region *(grey area)* and microbial inactivation levels for *B. cereus* and *E. coli* (95% upper probability interval) are indicated for orientation (van Asselt and Zwietering 2006).

10.3 Supplementary note III

Direct heat treatment of concentrated milk is promising as it facilitates the maximization of microbial effects without coagulation due to the small difference in the temperature sensitivity of coagulation and inactivation reactions. Negligible come-up times and cooling times also facilitate the estimation of possible heating times. Then calculations described in section 7.2 are sufficient.

Indirect heat treatment is still more frequently used for dairy products. Therefore, an attempt was made to calculate critical heating conditions for indirect heat treatment of concentrated skim milk by continuous integration of temperature-time profiles. The overall heat load is calculated by integration of the rate constants over time. In the case of the Weibullian model, the characteristic time $\alpha_{w,T,s}$ is integrated over time.

For non-isothermal heat treatments of CSM, we can define the integral

$$\int_0^t \frac{1}{\alpha_{w,T,s}}\,dt = \int_0^t \frac{1}{\alpha_{w,T_{ref},s_{ref}} \cdot f_T \cdot f_s}\,dt \qquad (10.1)$$

which becomes

$$\int_0^t \frac{1}{\alpha_{w,T,s}}\,dt = \frac{1}{\alpha_{w,T_{ref},s_{ref}} \cdot f_s}\int_0^t \frac{1}{f_T}\,dt = \frac{1}{\alpha_{w,s}}\int_0^t \frac{1}{f_T}\,dt \tag{10.2}$$

when the total solids is constant during the heat treatment. Calculated data for f_T and f_s is shown in Fig. 10-3. Thereby, a heating temperature of 116 °C and a CSM total solids content of 27% total solids were taken as reference points where $f_s = f_T = 1$.

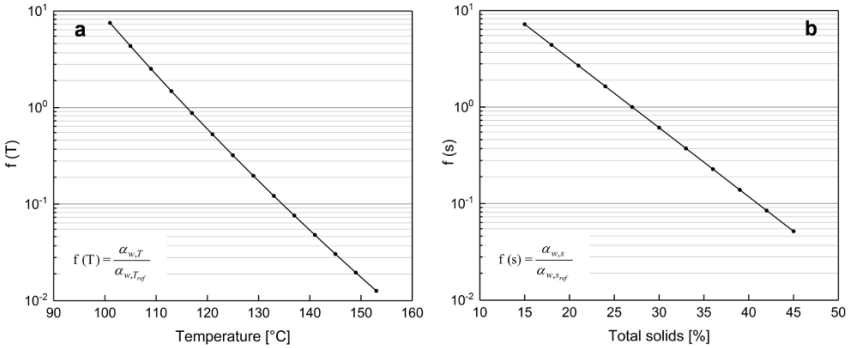

Fig. 10-3: Calculated data for the factor f_T depending on temperature (a) and f_s depending on total solids content of CSM (b).

We can arbitrarily define 2% sedimentable protein of total protein as the critical or acceptable level in terms of overall possible heat load on the concentrate of a certain total solids content. Then the integral in eq. 10.2 should not exceed 0.115 which can be defined as critical degree of aggregation A* equal to unity in accordance with the standardized values of B* and C*.

$$A^* = \frac{1}{0.115 \cdot \alpha_{w,s}}\int_0^t \frac{1}{f_T}\,dt \approx \frac{8.7}{\alpha_{w,s}}\int_0^t \frac{1}{f_T}\,dt \le 1 \tag{10.3}$$

A^* can be defined for a certain CSM total solids content and calculated by discrete integration using Fig. 7-6 (Fig. 9-2) as A_s^* which is shown in eq. 10.4

$$A_s^* = \int_0^t \frac{1}{t_{R,\ A_s^*=1}}\,dt \approx \sum_0^t \frac{\Delta t}{t_{R,A_s^*=1}} \le 1 \tag{10.4}$$

when $t_{R,\ A_s^*=1}$ is defined as the reliable lifetime for a certain total solids content resulting in a maximum of 2% of sedimentable protein of total protein. A relative amount of 5 and 10% of sedimentable protein corresponds to $A^* = 1.9$ and $A^* = 3.1$, respectively. Then Fig. 10-2 can be used to perform the integration.

References

Albert I, Mafart P (2005) A modified Weibull model for bacterial inactivation. Int J Food Microbiol 100:197–211

Andritsos N, Yiantsios SG, Karabelas AJ (2002) Calcium phosphate scale formation from simulated milk ultrafiltrate solutions. Food Bioprod Process 80:223–230

Anema SG (1998) Effect of milk concentration on heat-induced, pH-dependent dissociation of casein from micelles in reconstituted skim milk at temperatures between 20 and 120 °C. J Agric Food Chem 46:2299–2305

Anema SG (2008) On heating milk, the dissociation of kappa-casein from the casein micelles can precede interactions with the denatured whey proteins. J Dairy Res 75:415–421

Anema SG (2009) Effect of milk solids concentration on the pH, soluble calcium and soluble phosphate levels of milk during heating. Dairy Sci & Technol 89:501–510

Anema SG, Klostermeyer H (1996) ζ-Potentials of casein micelles from reconstituted skim milk heated at 120 °C. Int Dairy J 6:673–687

Anema SG, Klostermeyer H (1997) Heat-induced, pH-dependent dissociation of casein micelles on heating reconstituted skim milk at temperatures below 100 °C. J Agric Food Chem 45:1108–1115

Anema SG, Li Y (2000) Further studies on the heat-induced, pH-dependent dissociation of casein from the micelles in reconstituted skim milk. LWT - Food Sci Technol 33:335–343

Anema SG, Li Y (2003a) Association of denatured whey proteins with casein micelles in heated reconstituted skim milk and its effect on casein micelle size. J Dairy Res 70:73–83

Anema SG, Li Y (2003b) Effect of pH on the association of denatured whey proteins with casein micelles in heated reconstituted skim milk. J Agric Food Chem 51:1640–1646

Anema SG, Lowe EK, Lee SK (2004) Effect of pH at heating on the acid-induced aggregation of casein micelles in reconstituted skim milk. LWT - Food Sci Technol 37:779–787

Aoki T, Hatanaka C, Imamura T (1977) Large casein micelles and soluble casein in heated concentrated whey protein-free milk. Agric Biol Chem 41:2349–2355

Aoki T, Kako Y (1983) Relation between micelle size and formation of soluble casein on heating concentrated milk. J Dairy Res 50:207

Aoki T, Suzuki H, Imamura T (1974) Formation of soluble casein in whey protein-free milk heated at high temperature. Milchwiss 29:589–594

Aoki T, Suzuki H, Imamura T (1975) Some properties of soluble casein in heated concentrated whey protein-free milk. Milchwiss 30:30–35

Aoki T, Umeda T, Kako Y (1990) Cleavage of the linkage between colloidal calcium phosphate and casein on heating milk at high temperature. J Dairy Res 57:349–354

Augustin MA, Clarke PT (1990) Effects of added salts on the heat stability of recombined concentrated milk. J Dairy Res 57:213–226

Barsa CS, Normand MD, Peleg M (2012) On models of the temperature effect on the rate of chemical reactions and biological processes in foods. Food Eng Rev 4:191–202

Bastian ED, Brown RJ (1996) Plasmin in milk and dairy products: An update. Int Dairy J 6:435–457

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7

Belec J, Jenness R (1962) Dephosphorization of casein by heat treatment. II. In skimmilks. J Dairy Sci 45:20–26

Beliciu CM, Moraru CI (2009) Effect of solvent and temperature on the size distribution of casein micelles measured by dynamic light scattering. J Dairy Sci 92:1829–1839

Bendicho S, Barbosa-Canovas GV, Martin O (2003) Reduction of protease activity in simulated milk ultrafiltrate by continuous flow high intensity pulsed electric field treatments. J Food Science 68:952–957

Bobe G, Beitz DC, Freeman AE, Lindberg GL (1998) Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. J Agric Food Chem 46:458–463

Boitz LI, Mayer HK (2015) Evaluation of furosine, lactulose and acid-soluble β-lactoglobulin as time temperature integrators for whipping cream samples at retail in Austria. Int Dairy J 50:24–31

Bonfatti V, Grigoletto L, Cecchinato A, Gallo L, Carnier P (2008) Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants. J Chromatogr A 1195:101–106

Bonizzi I, Buffoni JN, Feligini M (2009) Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. J Chromatogr A 1216:165–168

Boumpa T, Tsioulpas A, Grandison AS, Lewis MJ (2008) Effects of phosphates and citrates on sediment formation in UHT goats' milk. J Dairy Res 75:160–166

Bulca S, Dumpler J, Kulozik U (2016) Kinetic description of heat-induced cross-linking reactions of whey protein-free casein solutions. Int J Dairy Technol 69:489–496

Burton H (1984) Reviews of the progress of dairy science: The bacteriological, chemical, biochemical, and physical changes that occur in milk at temperatures of 100-150°C. J Dairy Res 51:341–363

Burton H (1994) Ultra-high-temperature processing of milk and milk products. Springer, New York

Carić M, Akkerman JC, Milanovic´ S., Kentish SE (2009) Technology of evaporators, membrane processing and dryers. In: Tamime AY (ed) Dairy powders and concentrated milk products, 1st ed. Blackwell, Ames, pp 99–148

Carroll RJ, Thompson MP, Melnychyn P (1971) Gelation of concentrated skimmilk: Electron microscopic study. J Dairy Sci 54:1245–1252

Chandrapala J, McKinnon IR, Augustin MA, Udabage P (2010) The influence of milk composition on pH and calcium activity measured in situ during heat treatment of reconstituted skim milk. J Dairy Res 77:257–264

Chavan RS, Khedkar CD, Jana AH (2011) UHT milk processing and effect of plasmin activity on shelf life: A review. Comp Rev Food Sci Food Safety 10:251–268

Claeys WL, van Loey AM, Hendrickx ME (2003) Kinetics of hydroxymethylfurfural, lactulose and furosine formation in milk with different fat content. J Dairy Res 70:85–90

Cole WC, Tarassuk NP (1946) Heat coagulation of milk. J Dairy Sci 29:421–429

Corradini MG, Normand MD, Peleg M (2006) Expressing the equivalence of non-isothermal and isothermal heat sterilization processes. J Sci Food Agric 86:785–792

Creamer LK, Berry GP, Mills OE (1977) A study of the dissociation of β-casein from the bovine casein micelle at low temperature. New Zeal J Dairy Sci Technol 12:58–66

Crowley SV, Boudin M, Chen BY, Gazi I, Huppertz T, Kelly AL, O'Mahony JA (2015) Stability of milk protein concentrate suspensions to in-container sterilisation heating conditions. Int Dairy J 50:45–49

Crowley SV, Megemont M, Gazi I, Kelly AL, Huppertz T, O'Mahony JA (2014) Heat stability of reconstituted milk protein concentrate powders. Int Dairy J 37:104–110

Curley DM, Kumosinski TF, Unruh JJ, Farrell Jr. HM (1998) Changes in the secondary structure of bovine casein by Fourier transform infrared spectroscopy: Effects of calcium and temperature. J Dairy Sci 81:3154–3162

Dalgleish DG (2011) On the structural models of bovine casein micelles—review and possible improvements. Soft Matter 7:2265–2272

Dalgleish DG, Corredig M (2012a) The structure of the casein micelle of milk and its changes during processing. Ann Rev Food Sci Technol 3:449–467

Dalgleish DG, Corredig M (2012b) The structure of the casein micelle of milk and its changes during processing. Ann Rev Food Sci Technol 3:449–467

Dalgleish DG, Pouliot Y, Paquin P (1987) Studies on the heat stability of milk: II. Association and dissociation of particles and the effect of added urea. J Dairy Res 54:39–49

Dalgleish DG, Spagnuolo PA, Douglas Goff H (2004) A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. Int Dairy J 14:1025–1031

Dannenberg F, Kessler H-G (1988) Reaction kinetics of the denaturation of whey proteins in milk. J Food Sci 53:258–263

Darling DF (1980) Heat stability of milk. J Dairy Res 47:199–210

Davies DT, White JCD (1966) The stability of milk protein to heat: I. Subjective measurement of heat stability of milk. J Dairy Res 33:67–81

de Jong P (2008) Thermal processing of milk. In: Britz TJ, Robinson RK (eds) Advanced dairy science and technology. Blackwell, Oxford, pp 1–34

de Kort E, Minor M, Snoeren T, Hooijdonk T, Linden E (2009) Calcium-binding capacity of organic and inorganic ortho- and polyphosphates. Dairy Sci & Technol 89:283–299

de Kort E, Minor M, Snoeren T, van Hooijdonk T, van der Linden E (2012) Effect of calcium chelators on heat coagulation and heat-induced changes of concentrated micellar casein solutions: The role of calcium-ion activity and micellar integrity. Int Dairy J 26:112–119

de Kruif CG, Huppertz T, Urban VS, Petukhov AV (2012) Casein micelles and their internal structure. Adv Colloid Interfac 171–172:36–52

de Wit JN, Klarenbeek G, Graaf C de (1986) The Klaro-Graph: a new instrument for measuring viscosity, density and heat stability of milk and milk concentrates at temperatures up to 140 degrees. Voedingsmiddelentechnologie 19:25–27

Depping V, Grunow M, van Middelaar C, Dumpler J (2017) Integrating environmental assessment in new product development and processing technology selection: milk concentrates as substitutes for milk powders. J Clean Prod 149:1–10

Deysher EF, Webb BH, Holm GE (1929) The relations of temperature and time of forewarming of milk to the heat stability of its evaporated product. J Dairy Sci 12:80–89

Dogan Z, Weidendorfer K, Müller-Merbach M, Lembke F, Hinrichs J (2009) Inactivation kinetics of Bacillus spores in batch- and continuous-heating systems. LWT - Food Sci Technol 42:81–86

Donato L, Dalgleish DG (2006) Effect of the pH of heating on the qualitative and quantitative compositions of the sera of reconstituted skim milks and on the mechanisms of formation of soluble aggregates. J Agric Food Chem 54:7804–7811

Donato L, Guyomarc'h F (2009) Formation and properties of the whey protein/κ-casein complexes in heated skim milk — A review. Dairy Sci & Technol 89:3–29

Dumpler J, Kieferle I, Wohlschläger H, Kulozik U (2017a) Milk ultrafiltrate analysis by ion chromatography and calcium activity for SMUF preparation for different scientific purposes and prediction of its supersaturation. Int Dairy J 68:60–69

Dumpler J, Kulozik U (2015) Heat stability of concentrated skim milk as a function of heating time and temperature on a laboratory scale – Improved methodology and kinetic relationship. Int Dairy J 49:111–117

Dumpler J, Kulozik U (2016) Heat-induced coagulation of concentrated skim milk heated by direct steam injection. Int Dairy J 59:62–71

Dumpler J, Peraus F, Depping V, Stefánsdóttir B, Grunow M, Kulozik U (2017b) Modelling of heat stability and heat-induced aggregation of casein micelles in concentrated skim milk using a Weibullian model. Int J Dairy Technol submitted

Dumpler J, Wohlschläger H, Kulozik U (2017c) Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. A selection of papers from the 6th International Symposium of Dairy Dried Products (SDDP), Dublin, 11-13th April 2016. Dairy Sci & Technol 96:807–826

Elliott AJ, Datta N, Amenu B, Deeth HC (2005) Heat-induced and other chemical changes in commercial UHT milks. J Dairy Res 72:442–446

Faka M, Lewis MJ, Grandison AS, Deeth H (2009) The effect of free Ca^{2+} on the heat stability and other characteristics of low-heat skim milk powder. Int Dairy J 19:386–392

FAO (2016) The global dairy sector: Facts. http://www.fil-idf.org/wp-content/uploads/2016/12/FAO-Global-Facts-1.pdf. Accessed 19 April 2017

Farrell GC, Allgood RG (1970) Notes on the method for determining the heat stability of skim milk powder. Austr J Dairy Technol 25:121–122

Farrell Jr. HM, Wickham ED, Unruh JJ, Qi PX, Hoagland PD (2001) Secondary structural studies of bovine caseins: temperature dependence of β-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. 5th International Hydrocolloids Conference. Food Hydrocolloid 15:341–354

Ferrer M, Alexander M, Corredig M (2014) Changes in the physico-chemical properties of casein micelles during ultrafiltration combined with diafiltration. LWT - Food Sci Technol 59:173–180

Fink R, Kessler H-G (1986) HMF values in heat treated and stored milk. Milchwiss 41:638–641

Foissy H, Kneifel W (1984) An automatic method for measuring the heat coagulation time of milk powder solutions. J Dairy Res 51:325–329

Fox PF (1981) Heat-induced changes in milk preceding coagulation. J Dairy Sci 64:2127–2137

Fox PF, Hearn CM (1978a) Heat stability of milk: influence of denaturable proteins and detergents on pH sensitivity. J Dairy Res 45:159–172

Fox PF, Hearn CM (1978b) Heat stability of milk: influence of dilution and dialysis against water. J Dairy Res 45:149–157

Fox PF, Hearn CM (1978c) Heat stability of milk: influence of κ-casein hydrolysis. J Dairy Res 45:173–181

Fox PF, Hoynes MCT (1975) Heat stability of milk: influence of colloidal calcium phosphate and β-lactoglobulin. J Dairy Res 42:427–435

Fox PF, Uniacke-Lowe T, McSweeney PLH, O'Mahony JA (2015) Dairy chemistry and biochemistry, 2nd ed. Springer, New York

Frèche M, Heughebaert JC (1989) Calcium phosphate precipitation in the 60–80°C range. J Cryst Growth 94:947–954

Gao R, Halsema FED, Temminghoff EJM, van Leeuwen HP, van Valenberg HJF, Eisner MD, Giesbers M, van Boekel MAJS (2010a) Modelling ion composition in simulated milk ultrafiltrate (SMUF). I: Influence of calcium phosphate precipitation. Food Chem 122:700–709

Gao R, van Halsema F, Temminghoff E, van Leeuwen HP, van Valenberg H, Eisner MD, van Boekel MAJS (2010b) Modelling ion composition in simulated milk ultrafiltrate (SMUF) II. Influence of pH, ionic strength and polyphosphates. Food Chem 122:710–715

Gao R, van Leeuwen HP, Temminghoff EJ, van Valenberg HJ, Eisner MD, van Boekel MAJS (2010c) Effect of disaccharides on ion properties in milk-based systems. J Agric Food Chem 58:6449–6457

Gao R, van Leeuwen HP, van Valenberg H, van Boekel MAJS (2011) Accurate determination of the Ca^{2+} activity in milk-based systems by Ca-ISE: Effects of ionic composition on the single Ca^{2+} activity coefficient and liquid junction potentials. Food Chem 129:619–623

Gaucheron F (2005) The minerals of milk. Reprod Nutr Dev 45:473–483

Gaucheron F (2011) Milk and dairy products: a unique micronutrient combination. J Am Coll Nutr 30:400S–409S

Gonzalez-Jordan A, Thomar P, Nicolai T, Dittmer J (2015) The effect of pH on the structure and phosphate mobility of casein micelles in aqueous solution. Food Hydrocolloid 51:88–94

Greene RF, Pace CN (1974) Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α-chymotrypsin, and β-lactoglobulin. J Biol Chem 249:5388–5393

Hardy EE, Muir DD, Sweetsur AWM, West IGN (1984) Changes of calcium phosphate partition and heat stability during manufacture of sterilized concentrated milk. J Dairy Sci 67:1666–1673

Henle T, Schwarzenbolz UP, Klostermeyer H (1996) Irreversible crosslinking of casein during storage of UHT-treated skim milk. In: International Dairy Federation (ed) Heat treatments & alternative

methods: Proceedings of the IDF symposium held in Vienna (Austria), 6 - 8 September 1995, Brussels, pp 290–298
Hinrichs J (2000) UHT processed milk concentrates. Lait 80:15–23
Hinrichs J, Boheim C, Kessler H-G (1998) UHT-Erhitzen von Nanofiltrations-Milchkonzentraten. DMW 49:307–309
Holm GE, Deysher EF, Evans FR (1923) The relationships of concentration and time to the temperature of coagulation of evaporated skim and whole milk. J Dairy Sci 6:556–568
Holm GE, Webb BH, Deysher EF (1932) The heat coagulation of milk: 1.-Variations in the compositions, heat stability, and other tests of milks from cows during the course of a lactation period. J Dairy Sci 15:331–343
Holt C (1982) Inorganic constituents of milk III. The colloidal calcium phosphate of cow's milk. J Dairy Res 49:29–38
Holt C (1995) Effect of heating and cooling on the milk salts and their interaction with casein. In: Fox PF (ed) Heat-induced changes in milk, 2. ed. International Dairy Federation, Brussels, Belgium, pp 105–133
Holt C, Carver JA, Ecroyd H, Thorn DC (2013) Invited review: Caseins and the casein micelle: Their biological functions, structures, and behavior in foods. J Dairy Sci 96:6127–6146
Holt C, Davies DT, Law AJR (1986) Effects of colloidal calcium phosphate content and free calcium ion concentration in the milk serum on the dissociation of bovine casein micelles. J Dairy Res 53:557–572
Holt C, Horne DS (1996) The hairy casein micelle: Evolution of the concept and its implications for dairy technology. Neth Milk Dairy J 50:85–111
Holt C, Muir DD, Sweetsur AWM (1978a) Seasonal changes in the heat stability of milk from creamery silos in south-west Scotland. J Dairy Res 45:183–190
Holt C, Muir DD, Sweetsur AWM (1978b) The heat stability of milk and concentrated milk containing added aldehydes and sugars. J Dairy Res 45:47–52
Holt C, Sawyer L (1993) Caseins as rheomorphic proteins: Interpretation of primary and secondary structures of the αs1-, β- and κ-caseins. Journal of the Chemical Society, Faraday Transactions 89:2683–2692
Holt C, Timmins PA, Errington N, Leaver J (1998) A core-shell model of calcium phosphate nanoclusters stabilized by β-casein phosphopeptides, derived from sedimentation equilibrium and small-angle X-ray and neutron-scattering measurements. Eur J Biochem 252:73–78
Horak FP (1980) Über die Reaktionskinetik der Sporenabtötung und chemischer Veränderungen bei der thermischen Haltbarmachung von Milch zur Optimierung von Erhitzungsverfahren. Dissertation, Chair for Food Process Engineering and Dairy Technology
Horne DS (1992) Ethanol stability. In: Fox PF (ed) Advanced Dairy Chemistry: Volume 1 - Proteins, 1st edn. Elsevier Applied Science, London, pp 657–690
Horne DS (1998) Casein interactions: Casting light on the black boxes, the structure in dairy products. Int Dairy J 8:171–177
Horne DS (2006) Casein micelle structure: Models and muddles. Current Opinion in Colloid & Interface Science 11:148–153
Horne DS, Muir DD (1990) Alcohol and heat stability of milk protein. J Dairy Sci 73:3613–3626
Hostettler H, Stein J, Imhof K (1968) On the viscosity and age-gelation of UHT sterilized evaporated milk during storage. Mitteilungen aus dem Gebiete der Lebensmitteluntersuchung und Hygiene 59:60–77
Hougaard AB, Hammershøj M, Vestergard JS, Poulsen OLE, Ipsen RH (2009) Instant infusion pasteurisation of bovine milk. I. Effects on bacterial inactivation and physical–chemical properties. Int J Dairy Technol 62:484–492
Huppertz T (2014) Heat stability of transglutaminase-treated milk. Int Dairy J 38:183–186
Huppertz T (2016) Heat stability of milk. In: McSweeney PLH, O'Mahony JA (eds) Advanced dairy chemistry: 1B. Applied aspects, 4th ed. Springer, New York, pp 179–196

Huppertz T, Grosman S, Fox PF, Kelly AL (2004) Heat and ethanol stabilities of high-pressure-treated bovine milk. Int Dairy J 14:125–133

Hurt EE, Barbano DM (2015) Factors that influence the membrane area of a multistage microfiltration process required to produce a micellar casein concentrate. J Dairy Sci 98:2222–2233

Hurt EE, Zulewska J, Newbold M, Barbano DM (2010) Micellar casein concentrate production with a 3X, 3-stage, uniform transmembrane pressure ceramic membrane process at 50°C. J Dairy Sci 93:5588–5600

Jenness R, Koops J (1962) Preparation and properites of a salt solution which simulates milk ultrafiltrate. Neth Milk Dairy J 16:153–164

Jeurnink TJM, de Kruif CG (1993) Changes in milk on heating: viscosity measurements. J Dairy Res 60:139

Kaombe DD, Du Y, Lewis MJ (2012) Mineral partitioning in milk and milk permeates at high temperature. J Dairy Res 79:1–6

Kasinos M, Tran Le T, van der Meeren P (2014) Improved heat stability of recombined evaporated milk emulsions upon addition of phospholipid enriched dairy by-products. Food Hydrocolloid 34:112–118

Kelly PM, Kelly J, Mehra R, Oldfield DJ, Raggett E, O'Kennedy BT (2000) Implementation of integrated membrane processes for pilot scale development of fractionated milk components. Lait 80:139–153

Kelly PM, Roefs SPFM (1990) Comparison of a Klarograph and oil-bath methods for the measurement of the heat stability of reconstituted milks. Irish J Food Sci Technol 14:132

Kessler H-G (1975) Technologischer Stand der H-Milch-Herstellung. Technological position of manufacturing milk with ultra-high temperature. Deutsche Molkerei-Zeitung 96:1646–1650

Kessler H-G (2002) Food and bio process engineering. Dairy technology, 5th rev. and extended edition. A. Kessler, Munich

Kessler H-G, Horak P (1981) Objective evaluation of UHT-milk-heating by standardization of bacteriological and chemical effects. Milchwiss 36:129–133

Kieseker FG, Aitken B (1988) An objective method for determination of heat-stability of milk powders. Austr J Dairy Technol 43:26–31

Kieseker FG, Clarke PT, Aitken B (1984) Comparison of recombination and reconstitution processes for the preparation of dairy products. Austr J Dairy Technol 39:145–153

Kiesner C, Hoffmann W, Buchheim W (1995) Pilotanlage zur Herstellung von Kondensmilch und anderen sterilen Milchkonzentraten nach einem modifizierten Verfahren. Kiel Milchw Forsch 47:35–44

Kneifel W, Foissy H, Goller H (1987) Comparison of the An Bord Bainne method, the Australian Standard method and an automatic procedure for determining the heat-coagulation time of 20% reconstituted milk. Int J Dairy Technol 40:42–45

Kowalik G, Tacke L, Schwenzow U (2007) Injektor und Verfahren zum Einleiten eines dampfförmigen Wärmeträgers in ein flüssiges Produkt (DE102007017704A1)

Kulozik U (1986) Einflüsse auf die Permeation von Wasser und gelösten Stoffen sowie auf den Deckschichtabtrag bei der Umkehrosmose. Dissertation, Technische Universität München

Kulozik U, Kersten M (2002) New ways for the fractionation of dairy minor constituents using UTP-membrane technology: Proceedings of the IDF World Dairy Summit 2001, New Zealand. In: International Dairy Federation (ed) New ways for the fractionation of dairy and minor constituents using UTP-membrane technology. IDF, Brussels, Belgium, pp 37–42

Kumosinski TF, Brown EM, Farrell HMJ (1991a) Three-dimensional molecular modeling of bovine caseins: α_{s1}-casein. J Dairy Sci 74:2889–2895

Kumosinski TF, Brown EM, Farrell HMJ (1991b) Three-dimensional molecular modeling of bovine caseins: κ-casein. J Dairy Sci 74:2879–2887

Kumosinski TF, Brown EM, Farrell HMJ (1993a) Three-dimensional molecular modeling of bovine caseins: A refined, energy-minimized κ-casein structure. J Dairy Sci 76:2507–2520

Kumosinski TF, Brown EM, Farrell HMJ (1993b) Three-dimensional molecular modeling of bovine caseins: an energy-minimized β-casein structure. J Dairy Sci 76:931–945

Kumosinski TF, Farrell HM, JR. (1991) Calcium-induced associations of the caseins: Thermodynamic linkage of calcium binding to colloidal stability of casein micelles. J Protein Chem 10:3–16

Le Ray C, Maubois J-L, Gaucheron F, Brule G, Pronnier P, Garnier F (1998) Heat stability of reconstituted casein micelle dispersions: Changes induced by salt addition. Lait 78:375–390

Lehmann L, Buckin V (2005) Determination of the heat stability profiles of concentrated milk and milk ingredients using high resolution ultrasonic spectroscopy. J Dairy Sci 88:3121–3129

Leviton A, Pallansch MJ (1961a) High temperature-short time sterilized evaporated milk. II. Laboratory techniques for the preparation and study of sterile evaporated milk. J Dairy Sci 44:442–450

Leviton A, Pallansch MJ (1961b) High-temperature short-time sterilized evaporated milk. III. The influence of the lipid phase on heat and storage stability. J Dairy Sci 44:633–643

Lewis MJ (2011) The measurement and significance of ionic calcium in milk - A review. Int J Dairy Technol 64:1–13

Lorient D (1979) Covalent bonds formed in proteins during milk sterilization: Studies on caseins and casein peptides. J Dairy Res 46:393–396

Loveday SM (2016) β-Lactoglobulin heat denaturation: A critical assessment of kinetic modelling. Int Dairy J 52:92–100

Lowe EK, Anema SG, Bienvenue A, Boland MJ, Creamer LK, Jiménez-Flores R (2004) Heat-induced redistribution of disulfide bonds in milk proteins. 2. Disulfide bonding patterns between bovine beta-lactoglobulin and kappa-casein. J Agric Food Chem 52:7669–7680

Mayer HK (2011) ESL-Milch mit übermäßiger Hitzebelastung? Analytik im Dienst des Verbraucherschutzes. GIT Labor-Fachzeitschrift 55:188–189

Mayer HK, Raba B, Meier J, Schmid A (2010) RP-HPLC analysis of furosine and acid-soluble β-lactoglobulin to assess the heat load of extended shelf life milk samples in Austria. Dairy Sci & Technol 90:413–428

Mayr R, Gutser K, Busse M, Seiler H (2004) Gram positive nonsporeforming recontaminants are frequent spoilage oranisms of German retail ESL (Extended shelf life) milk. Milchwiss 59:262–266

McCrae CH, Hirst D, Law AJR, Muir DD (1994) Heat stability of homogenized milk: role of interfacial protein. J Dairy Res 61:507–516

McCrae CH, Muir DD (1995) Heat stability of milk. In: International Dairy Federation (ed) Heat-induced changes in milk, 2nd edn., Brussels, pp 206–230

McMahon DJ, Oommen BS (2008) Supramolecular structure of the casein micelle. J Dairy Sci 91:1709–1721

McSweeney PLH, Fox PF (eds) (2013) Advanced dairy chemistry: 1A. Basic aspects, 4th ed. Springer, New York

Mekmene O, Leconte N, Rouillon T, Quillard S, Bouler JM, Gaucheron F (2012) Physicochemical characterisation of calcium phosphates prepared from milk ultrafiltrates: Effect of the mineral composition. Int J Dairy Technol 65:334–341

Melin T, Rautenbach R (2004) Membranverfahren: Grundlagen der Modul- und Anlagenauslegung, 2nd ed. Springer, Berlin, Heidelberg

Merkel BJ, Planer-Friedrich B (2008) Grundwasserchemie. Springer, Berlin, Heidelberg

Metwalli AAM, Metwalli NH, van Boekel MAJS (1996) Effect of urea on heat-induced changes in milk. Neth Milk Dairy J 50:427–457

Metwalli AAM, van Boekel MAJS (1996) Effect of urea on heat coagulation of milk. Neth Milk Dairy J 50:459–476

Miller PG, Sommer HH (1940) The coagulation temperature of milk as affected by pH, salts, evaporation and previous heat treatment. J Dairy Sci 23:405–421

Mora-Gutierrez A, Farrell HM, JR., Kumosinski TF (1993) Comparison of calcium-induced associations of bovine and caprine caseins and the relationship of α_{s1}-casein content to colloidal stabilization: A thermodynamic linkage analysis. J Dairy Sci 76:3690–3697

Morales F-J, Romero C, Jiménez-Pérez S (1995) New methodologies for kinetic study of 5-(Hydroxymethyl)-furfural formation and reactive lysine blockage in heat-treated milk and model systems. J Food Protection 58:310–315

Morales F-J, Romero C, Jiménez-Pérez S (2000) Characterization of industrial processed milk by analysis of heat-induced changes. Int J Food Sci Tech 35:193–200

Morrissey PA (1969) The heat stability of milk as affected by variations in pH and milk salts. J Dairy Res 36:343–351

Morrissey PA, O'Mahony F (1976) Heat stability of forewarmed milks: Influence of κ-casein, serum proteins and divalent cations. J Dairy Res 43:267–274

Mounsey JS, O'Kennedy BT (2009) Stability of β-lactoglobulin/micellar casein mixtures on heating in simulated milk ultrafiltrate at pH 6.0. Int J Dairy Technol 62:493–499

Muir DD (1984) UHT-sterilized milk concentrate: A review of practical methods of production. Int J Dairy Technol 37:135–141

Muir DD, Sweetsur AWM (1978) The effect of concentration on heat stability of skim-milk. J Dairy Res 45:37–45

Muir DD, Sweetsur AWM (1984) Optimization of the heat stability of protein-rich concentrates prepared by ultrafiltration of skim-milk. Int J Food Sci Tech 19:263–271

Muir DD, Sweetsur AWM, Holt C (1979) The synergic effect of urea and aldehydes on the heat stability of concentrated skim-milk. J Dairy Res 46:381–384

Muller LL (1982) Manufacture of casein, caseinates and co-precipitates. In: Fox PF (ed) Developments in dairy chemistry - 1 Proteins. Applied Science Publishers, London, New York, pp 315–337

Müller-Buschbaum P, Gebhardt R, Roth SV, Metwalli E, Doster W (2007) Effect of calcium concentration on the structure of casein micelles in thin films. Biophys J 93:960–968

Nangpal A, Reuter H (1990) Formation of lactulose during UHT treatment of milk - comparison between direct and indirect heating. Kiel Milchw Forsch 42:31–41

Newstead DF, Conaghan EF, Baldwin AJ (1979) Studies on the induction of heat stability in evaporated milk by preheating: Effects of milk concentration, homogenization and whey proteins. J Dairy Res 46:387–391

Nian Y, Chen BY, Aikman P, Grandison AS, Lewis MJ (2012) Naturally occurring variations in milk pH and ionic calcium and their effects on some properties and processing characteristics of milk. Int J Dairy Technol 65:490–497

Nieuwenhuijse JA, Sjollema A, van Boekel MAJS, van Vliet T (1991) The heat stability of concentrated skim milk. Neth Milk Dairy J 45:193–224

Nieuwenhuijse JA, Timmermans W, Walstra P (1988) Calcium and phosphate partitions during the manfacture of sterilized concentrated milk and their relations to the heat stability. Neth Milk Dairy J 42:387–421

Nieuwenhuijse JA, van Vliet T, Walstra P (1992) Kinetic aspects of the heat induced coagulation of concentrated skim milk. Neth Milk Dairy J 46:45–68

O'Connell JE, Fox PF (1999) Heat-induced changes in the calcium sensitivity of caseins. Int Dairy J 9:839–847

O'Connell JE, Fox PF (2000) The two-stage coagulation of milk proteins in the minimum of the heat coagulation time-pH profile of milk: Effect of casein micelle size. J Dairy Sci 83:378–386

O'Connell JE, Fox PF (2001) Effect of β-lactoglobulin and precipitation of calcium phosphate on the thermal coagulation of milk. J Dairy Res 68:81–94

Oldfield DJ, Singh H, Taylor MW, Pearce KN (2000) Heat-induced interactions of β-lactoglobulin and α-lactalbumin with the casein micelle in pH-adjusted skim milk. Int Dairy J 10:509–518

Olivares ML, Achkar NP, Zorrilla SE (2016) Rheological behavior of concentrated skim milk dispersions as affected by physicochemical conditions: change in pH and $CaCl_2$ addition. Dairy Sci & Technol:1–14

Omoarukhe ED, On-Nom N, Grandison AS, Lewis MJ (2010) Effects of different calcium salts on properties of milk related to heat stability. Int J Dairy Technol 63:504–511

On-Nom N, Grandison AS, Lewis MJ (2010) Measurement of ionic calcium, pH, and soluble divalent cations in milk at high temperature. J Dairy Sci 93:515–523

On-Nom N, Grandison AS, Lewis MJ (2012) Heat stability of milk supplemented with calcium chloride. J Dairy Sci 95:1623–1631

Pearce RJ (1979) Heat stability in concentrated and non-concentrated milks - the effect of urea and β-lactoglobulin levels and the influence of preheating. J Dairy Res 46:385–386

Peleg M, Corradini MG, Normand MD (2004) Kinetic Models of Complex Biochemical Reactions and Biological Processes. CIT 76:413–423

Pike ACW, Brew K, Acharya KR (1996) Crystal structures of guinea-pig, goat and bovine α-lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. Structure 4:691–703

Post AE, Arnold B, Weiss J, Hinrichs J (2012) Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of α_S- and β-casein. J Dairy Sci 95:1603–1616

Pouliot Y, Boulet M (1991) Seasonal variations in the heat stability of concentrated milk: Effect of added phosphates and pH adjustment. J Dairy Sci 74:1157–1162

Pouliot Y, Landry J, Giasson J (1991) Induction de la précipitation phosphocalcique dans le perméat de lactosérum de fromagerie. Lait 71:313–320

Pyne GT, McHenry KA (1955) The heat coagulation of milk. J Dairy Res 22:60

Ramasubramanian L, D'Arcy B, Deeth HC (2012) Heat-induced coagulation of whole milk by high levels of calcium chloride. Int J Dairy Technol 65:183–190

Rasmussen LK, Hojrup P, Peterson TE (1992) The multimeric structure and disulfide-bonding pattern of bovine kappa-casein. Eur J Biochem 207:215–222

Rauh VM, Johansen LB, Bakman M, Ipsen R, Paulsson M, Larsen LB, Hammershøj M (2015) Protein lactosylation in UHT milk during storage measured by liquid chromatography-mass spectrometry and quantification of furosine. Int J Dairy Technol 68:486–494

Rauh VM, Sundgren A, Bakman M, Ipsen R, Paulsson M, Larsen LB, Hammershøj M (2014) Plasmin activity as a possible cause for age gelation in UHT milk produced by direct steam infusion. Int Dairy J 38:199–207

Rice G, Barber A, O'Connor AJ, Stevens GW, Kentish SE (2010) A theoretical and experimental analysis of calcium speciation and precipitation in dairy ultrafiltration permeate. Int Dairy J 20:694–706

Robitaille G, Ayers C (1995) Effects of κ-casein glycosylation on heat stability of milk. Food Res Int 28:17–21

Rose D (1961a) Factors affecting the pH-sensitivity of the heat stability of milk from individual cows. J Dairy Sci 44:1405–1413

Rose D (1961b) β-Lactoglobulin and pH-sensitivity of the heat stability of evaporated milk. J Dairy Sci 44:1763

Rose D (1962) Factors affecting the heat stability of milk. J Dairy Sci 45:1305–1311

Rose D, Tessier H (1959) Composition of ultrafiltrates from milk heated at 80 to 230°F in relation to heat stability. J Dairy Sci 42:969–980

Rosmaninho R, Melo LF (2006) The effect of citrate on calcium phosphate deposition from simulated milk ultrafiltrate (SMUF) solution. J Food Eng 73:379–387

Saint Denis T, Humbert G, Gaillard J-L (2001) Heat inactivation of native plasmin, plasminogen and plasminogen activators in bovine milk: A revisited study. Lait 81:715–729

Sandra S, Dalgleish DG (2005) Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder. Int Dairy J 15:1095–1104

Sauer A, Moraru CI (2012) Heat stability of micellar casein concentrates as affected by temperature and pH. J Dairy Sci 95:6339–6350

Schmidt DG (1980) Colloidal aspects of casein. Neth Milk Dairy J 34:42–64

Schmitz-Schug I (2014) Improving the nutritional quality of dairy powders - analyzing and modeling lysine loss during spray drying as influenced by drying kinetics, thermal stress, physical state and molecular mobility, 1st edn. Dr. Hut, München

Schwermann S, Schwenzow U (2008a) Verfahrenskonzepte zur Herstellung von ESL-Milch: Teil 1: Grundsätztliches und direkte Erhitzungsverfahren. DMW 59:384–391

Schwermann S, Schwenzow U (2008b) Verfahrenskonzepte zur Herstellung von ESL-Milch: Teil 2: Indirekte Erhitzung mit einer UHT-Anlage. DMW 59:428–432

Sievanen K, Huppertz T, Kelly AL, Fox PF (2008) Influence of added calcium chloride on the heat stability of unconcentrated and concentrated bovine milk. Int J Dairy Technol 61:151–155

Sikand V, Tong PS, Walker J (2010) Heat stability of reconstituted, protein-standardized skim milk powders. J Dairy Sci 93:5561–5571

Singh G, Arora S, Sharma GS, Sindhu JS, Kansal VK, Sangwan RB (2007) Heat stability and calcium bioavailability of calcium-fortified milk. LWT - Food Sci Technol 40:625–631

Singh H (2004) Heat stability of milk. Int J Dairy Technol 57:111–119

Singh H, Creamer LK (1991) Influence of concentration of milk solids on the dissociation of micellar κ-casein on heating reconstituted milk at 120 °C. J Dairy Res 58:99–105

Singh H, Creamer LK (1992) Heat stability of milk. In: Fox PF (ed) Advanced Dairy Chemistry: Volume 1 - Proteins, 1st edn. Elsevier Applied Science, London, pp 621–656

Singh H, Creamer LK, Newstead DF (1995) Heat stability of concentrated milk. In: Fox PF (ed) Heat-induced changes in milk, 2. ed. International Dairy Federation, Brussels, Belgium, pp 256–278

Singh H, Fox PF (1985a) Heat stability of milk: pH-dependent dissociation of micellar κ-casein on heating milk at ultra high temperatures. J Dairy Res 52:529–538

Singh H, Fox PF (1985b) Heat stability of milk: the mechanism of stabilization by formaldehyde. J Dairy Res 52:65–76

Singh H, Fox PF (1986) Heat stability of milk: Further studies on the pH-dependent dissociation of micellar κ-casein. J Dairy Res 53:237–248

Singh H, Fox PF (1987a) Heat stability of milk: Influence of colloidal and soluble salts and protein modification on the pH-dependent dissociation of micellar κ-casein. J Dairy Res 54:523–534

Singh H, Fox PF (1987b) Heat stability of milk: Influence of modifying sulphydryl-disulphide interactions on the heat coagulation time–pH profile. J Dairy Res 54:347–359

Singh H, Fox PF (1987c) Heat stability of milk: Role of β-lactoglobulin in the pH-dependent dissociation of micellar κ-casein. J Dairy Res 54:509–521

Singh H, Latham JM (1993) Heat stability of milk: Aggregation and dissociation of protein at ultra-high temperatures. Int Dairy J 3:225–237

Singh H, Tokley RP (1990) Effects of preheat treatments and buttermilk addition on the seasonal variations in the heat stability of recombined evaporated milk and reconstituted concentrated milk. Austr J Dairy Technol 45:10–16

Slattery CW, Evard R (1973) A model for the formation and structure of casein micelles from subunits of variable composition. Biochim Biophys Acta 317:529–538

Smith JF, Malmgren B (1999) UHT RCM - UHT recombined concentrated milk. In: International Dairy Federation (ed) 3rd International Symposium on Recombined Milk & Milk Products: Special issue 9902. International Dairy Federation, Brussels, Belgium, pp 43–48

Sommer HH, Hart EB (1919) The heat coagulation of milk. J Biol Chem 40:137–151

Sommer HH, Hart EB (1922) The heat coagulation of milk. J Dairy Sci 5:525–543

Spanos N, Patis A, Kanellopoulou DG, Andritsos N, Koutsoukos PG (2007) Precipitation of calcium phosphate from simulated milk ultrafiltrate solutions. Crystal Growth & Design 7:25–29

Stoeckel M, Atamer Z, Hinrichs J (2014) Thermal inactivation of *Bacillus cereus* spores in micellar casein concentrates–effect of protein content and pH development. Dairy Sci & Technol 94:539–548

Sweetsur AWM, Muir DD (1980) Effect of concentration by ultrafiltration on the heat stability of skimmilk. J Dairy Res 47:327–335

Sweetsur AWM, Muir DD (1982) Natural variation in heat stability of concentrated milk before and after homogenization. Int J Dairy Technol 35:120–126

Sweetsur AWM, Muir DD (1983a) Effect of homogenization on the heat stability of milk. J Dairy Res 50:291–300

Sweetsur AWM, Muir DD (1983b) Influence of sulphydryl group interactions on the heat stability of homogenized concentrated milk. J Dairy Res 50:301–308
Sweetsur AWM, White JCD (1975) Studies on the heat stability of milk protein: III. Effect of heat-induced acidity in milk. J Dairy Res 42:73
Tan-Kintia RH, Fox PF (1999) Effect of various preheat treatments on the heat stability of unconcentrated milk. Int Dairy J 9:219–225
Taterka H, Castillo M (2015) The effect of whey protein denaturation on light backscatter and particle size of the casein micelle as a function of pH and heat-treatment temperature. Int Dairy J 48:53–59
Terebiznik MR, Jagus RJ, Cerrutti P, Huergo MS de, Pilosof MR (2000) Combined effect of nisin and pulsed electric fields on the inactivation of Escherichia coli. J Food Prot 63:741–746
Tessier H, Rose D (1961) Heat stability of casein in the presence of calcium and other salts. J Dairy Sci 44:1238–1246
Tessier H, Rose D (1964) Influence of κ-casein and β-lactoglobulin on the heat stability of skimmilk. J Dairy Sci 47:1047–1051
Tolkach A, Kulozik U (2006) Transport of whey proteins through 0.1 mm ceramic membrane: phenomena, modelling and consequences for concentration or diafiltration processes. Desalination 199:340–341
Töpel A (2016) Chemie und Physik der Milch: Naturstoff, Rohstoff, Lebensmittel, 4th ed. Behr's Verlag, Hamburg
Toro-Sierra J, Tolkach A, Kulozik U (2011) Fractionation of α-lactalbumin and β-lactoglobulin from whey protein isolate using selective thermal aggregation, an optimized membrane separation procedure and resolubilization techniques at pilot plant scale. Food Bioprocess Technol 6:1032–1043
Tran Le T, Saveyn P, Hoa HD, van der Meeren P (2008) Determination of heat-induced effects on the particle size distribution of casein micelles by dynamic light scattering and nanoparticle tracking analysis. Int Dairy J 18:1090–1096
Trinh B, Trinh KT, Haisman D (2007) Effect of total solids content and temperature on the rheological behaviour of reconstituted whole milk concentrates. J Dairy Res 74:116–123
van Asselt AJ, Sweere APJ, Rollema HS, de Jong P (2008) Extreme high-temperature treatment of milk with respect to plasmin inactivation. Int Dairy J 18:531–538
van Asselt ED, Zwietering MH (2006) A systematic approach to determine global thermal inactivation parameters for various food pathogens. Int J Food Microbiol 107:73–82
van Boekel MAJS (1998) Effect of heating on Maillard reactions in milk. Food Chem 62:403–414
van Boekel MAJS (2002) On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int J Food Microbiol 74:139–159
van Boekel MAJS (2009) Kinetic modeling of reactions in foods. Food science and technology. CRC Press, Boca Raton
van Boekel MAJS, Nieuwenhuijse JA, Walstra P (1989a) The heat coagulation of milk. 1. Mechanisms. Neth Milk Dairy J 43:97–127
van Boekel MAJS, Nieuwenhuijse JA, Walstra P (1989b) The heat coagulation of milk. 2. Kinetic studies. Neth Milk Dairy J 43:129–146
van Boekel MAJS, Walstra P (1995) Use of kinetics in studying heat-induced changes in food. In: International Dairy Federation (ed) Heat-induced changes in milk, 2nd edn., Brussels, pp 22–50
van Dijk HJM, Hersevoort A (1992) The properties of casein micelles. 5. The determination of heat-induced calcium phosphate precipitations in milk. Neth Milk Dairy J 46:69–76
van Kemenade MJJM, de Bruyn PL (1987) A kinetic study of precipitation from supersaturated calcium phosphate solutions. J Colloid Interface Sci 118:564–585
Visser S, Slangen CJ, Rollema HS (1991) Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. J Chromatogr A 548:361–370
Vyas HK, Tong PS (2004) Impact of source and level of calcium fortification on the heat stability of reconstituted skim milk powder. J Dairy Sci 87:1177–1180
Walstra P (1990) On the stability of casein micelles. J Dairy Sci 73:1965–1979

Walstra P (1999) Casein sub-micelles: Do they exist? Int Dairy J 9:189–192
Walstra P, Jenness R, Badings HT (1984) Dairy chemistry and physics. Wiley, New York
Webb BH, Bell RW, Deysher EF, Holm GE (1943) The effect of various degrees of forewarming upon the heat stability of milks of different solids concentrations. J Dairy Sci 26:571–578
Webb BH, Holm GE (1932) The heat coagulation of milk: II. The influence of various added salts upon the heat stabilities of milks of different concentrations. J Dairy Sci 15:345–366
White JCD, Davies DT (1958) 715. The relation between the chemical composition of milk and the stability of the caseinate complex: IV. Coagulation by heat. J Dairy Res 25:281
White JCD, Davies DT (1966) The stability of milk protein to heat: III. Objective measurement of heat stability of milk. J Dairy Res 33:93–102
White JCD, Sweetsur AWM (1977) Kinetics of the heat-induced aggregation of milk protein. J Dairy Res 44:237–247
Whiteley AJ, Muir DD (1996) Heat stability of homogenized concentrated milk: 1. Comparison of Microfuidizer with a valve homogenizer. Milchwiss 51:320–323
Whitney RM, Paulson K, Murthy GK (1952) A test of the heat stability of milk proteins for use in research. J Dairy Sci 35:937–948
Zin El-Din M, Aoki T (1993) Polymerization of casein on heating milk. Int Dairy J 3:581–588

Appendix

Peer reviewed publications

Publications included in this thesis

Dumpler J, Kulozik U (2015) Heat stability of concentrated skim milk as a function of heating time and temperature on a laboratory scale – Improved methodology and kinetic relationship. Int Dairy J 49:111–117. doi:10.1016/j.idairyj.2015.05.005

Dumpler J, Kulozik U (2016) Heat-induced coagulation of concentrated skim milk heated by direct steam injection. Int Dairy J 59:62–71. doi:10.1016/j.idairyj.2016.03.009

Dumpler J, Wohlschläger H, Kulozik U (2017) Dissociation and coagulation of caseins and whey proteins in concentrated skim milk heated by direct steam injection. Dairy Sci & Technol 96:807-826. doi:10.1007/s13594-016-0304-3

Dumpler J, Wohlschläger H, Kulozik U (2017) Milk ultrafiltrate analysis by ion chromatography and calcium activity for SMUF preparation for different scientific purposes and prediction of its supersaturation. Int Dairy J 68:60–69. doi:10.1016/j.idairyj.2016.12.009

Dumpler J, Peraus F, Depping V, Stefánsdóttir B, Grunow M, Kulozik U (2017) Modelling of heat stability and heat-induced aggregation of casein micelles in concentrated skim milk using a Weibullian model. Int J Dairy Technol **submitted**

Dumpler J, Dörle M-Th, Kulozik U (2017) Implication of pH and soluble calcium on micelle size and dissociation of κ-casein in relation to heat stability of micellar casein. LWT-Food Sci Technol **submitted**

Publications not included in this thesis

Kirchner B, Pfaffl MW, Dumpler J, von Mutius E, Ege MJ (2016) microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma. J Allergy Clin Immunol, 137:1893-1895. doi:10.1016/j.jaci.2015.10.028

J. Dumpler, *Heat Stability of Concentrated Milk Systems*,
https://doi.org/10.1007/978-3-658-19696-7

Bulca S, Dumpler J, Kulozik, U (2016) Kinetic description of heat-induced cross-linking reactions of whey protein-free casein solutions. Int J Dairy Technol 69:489-496. doi:10.1111/1471-0307.12357

Depping V, Grunow M, van Middelaar C, Dumpler J (2016) Integrating environmental assessment in new product development and processing technology selection: milk concentrates as substitutes for milk powders. J Cleaner Prod 149:1-10. doi:10.1016/j.jclepro.2017.02.070

Non reviewed publications

Dumpler J, Marx M (2012) Energieeinsparung durch haltbare Milch- und Molkekonzentrate. Jahresbericht 2012 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München, pp. 100-102

Dumpler J (2013) Einfluss des Trockenmassegehaltes von Umkehrosmose-Magermilch-konzentraten auf funktionelle Qualitätskriterien. Jahresbericht 2013 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München, pp. 86-88

Dumpler J, Helgert L (2014) Nutzung der Vorerhitzung zur gezielten Steigerung der Hitzestabilität von Magermilch-konzentrat unterschiedlicher Trockenmasse. Jahresbericht 2014 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München, pp. 90-92

Dumpler J, Peraus F (2015) Ansätze zur reaktionskinetischen Beschreibung der hitzeinduzierten Aggregation der Proteine in Magermilchkonzentrat. Jahresbericht 2015 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München, pp. 92-95

Marx M, Dumpler J, Kulozik U, Depping V, Stefánsdóttir B, Grunow M (2016) Milch und Molkenkonzentrate - Ökologisch und ökonomisch sinnvoll – Teil 1. Molkereiindustrie, August 2016, pp. 35-37

Marx M, Dumpler J, Kulozik U, Depping V, Stefánsdóttir B, Grunow M (2016) Milch und Molkenkonzentrate - Ökologisch und ökonomisch sinnvoll – Teil 2. Molkereiindustrie, September 2016, pp. 46-48

Dumpler J, Birkle P (2016) Bestimmung der Hitzestabilität von micellarem Caseinkonzentrat in Abhängigkeit von pH-Wert, Protein- und Molkenproteingehalt. Jahresbericht 2016 der milchwissenschaftlichen Forschungseinheiten am ZIEL, Technische Universität München, pp. 82-85

Dumpler J, Marx M, Kulozik U, Depping V, Stefánsdóttir B, Grunow, M (2017) Ökonomisch und ökologisch sinnvoll – Ressourcenschonende Produktion von Milch- und Molkekonzentraten. Lebensmitteltechnik 49(3):40-41.

Oral presentations[12]

Dumpler J, Kulozik U (2012). Grundlagen und Mechanismen der Strukturbildung bei der Gelbildung durch Proteine und Hydrokolloide. Technologieseminar 2012 - Verfahrens- und Strukturoptimierung in der Lebensmittelherstellung, Freising/Weihenstephan, October 25-26

Dumpler J, Kulozik U (2013) Heat stability of concentrated skim milk as a function of pH. Dairy Conference 2013, Universität Hohenheim/Stuttgart, September 16-17

Marx M, Dumpler J, Kulozik U (2013) Neue Erkenntnisse zur Erhitzung und Stabilität von Milch- und Molkenkonzentraten. Milchwissenschaftliche Herbsttagung, Freising/Weihenstephan, October 10-11

Dumpler J, Kulozik U (2014) Kristallisation von Calciumphosphat aus Ultrafiltrationspermeaten. Jahrestreffen der ProcessNet-Fachgruppen Lebensmittelverfahrenstechnik und Phytoextrakte, TU München, Freising/Weihenstephan, February 26-28

Dumpler J, Kulozik U (2014) „Quo vadis Magermilchkonzentrat?“ Lösungsansätze für (ultra)hocherhitzbare Magermilchkonzentrate hoher Trockenmasse. Milchwissenschaftliche Herbsttagung, Freising/Weihenstephan, October 9-11

Kulozik U, Marx M, Dumpler J (2014) Neue ressourcen- und energiesparende Prozesse zur Herstellung, Vertrieb/Logistik und Einsatz von Milch- und Molkekonzentraten als Ersatz für Milch- und Molkepulver. BLE Innovationstage 2014 - Neue Ideen für den Markt, Bonn, October 15-16

Dumpler J, Kulozik U (2015) Kinetic description of UHT induced aggregation in milk concentrates - Comparison of direct steam injection and lab scale indirect heating. ICEF 12 - International Congress on Engineering and Food, Québec City, Canada, June 14-18

Heidebrecht HJ, Hartinger M, Dumpler J, Kulozik U (2015) Assessment of polymeric spiral-wound membranes for milk protein fractionation, Euromembrane, Aachen, September 6-10

Dumpler J, Kulozik U (2015) Novel technologies for skim milk concentrates. 1. Chemical and physical means to prevent heat-induced aggregation of casein micelles in concentrated skim milk. Seminar on Emerging Dairy Technologies, Freising/Weihenstephan, September 16–18

Dumpler J, Kulozik U (2015) Novel technologies for skim milk concentrates. 2. Energy saving by thermal preservation and shelf life of concentrated skim milk as an alternative to drying. Seminar on Emerging Dairy Technologies, Freising/Weihenstephan, September 16–18

12 The presenting author(s) is (are) underlined.

Dumpler J, Kulozik U (2015) Reaction kinetics of heat-induced aggregation in skim milk concentrates - Comparison of lab scale indirect heating and direct steam injection. 9th NIZO Dairy Conference, Papendal, The Netherlands, September 30-October 2

Dumpler J, Kulozik U (2016) Reaction kinetics of heat-induced aggregation in skim milk concentrates - Comparison of lab scale indirect heating and direct steam injection. IDF Parallel Symposia. Dairy Products Concentration and Drying, Dublin, Ireland, April 11 – 14

Dumpler J, Marx M, Kulozik U, Depping V, Stefánsdóttir B, Grunow M (2016) Ressourcenschonende Produktion von Milcherzeugnissen am Beispiel von Milch- und Molkenkonzentrat. Tag der Studienfakultät Brau- und Lebensmitteltechnologie. Freising/Weihenstephan, June 24

Dumpler J, Kulozik U (2016) Milk concentrates as alternative to powder manufacture – Technical solutions for preservation with reduced economic and environmental impact. Seminar on Emerging Dairy Technologies, Freising/Weihenstephan, September 14-16

Dumpler J, Kulozik U (2016) Diafiltration and heat treatment of micellar casein concentrates – Factors affecting filtration performance and heat stability. Seminar on Emerging Dairy Technologies, Freising/Weihenstephan, September 14-16

Dumpler J, Marx M, Kulozik U, Depping V, Stefánsdóttir B, Grunow M (2016) Technologische, ökonomische und ökologische Aspekte ressourcenschonender Produktion von Milch- und Molkenkonzentrat. GDL Kongress Lebensmitteltechnologie 2016. Lemgo, October 20-22

Dumpler J, Marx M, Kulozik U, Depping V, Stefánsdóttir B, Grunow M (2016) Milk and whey concentrates vs. dairy powders – Opportunities and challenges towards a reduced environmental impact of dairy processing. 30th EFFoST International Conference. Vienna, Austria, November 28-30

Poster presentations[13]

Dumpler J, Kulozik U (2014) Changes in the composition of milk serum and its influence on heat stability of concentrated milk. 15th Food Colloids Conference, Karlsruhe, April 13-16

Heidebrecht HJ, Dumpler J, Kulozik U (2015) Thermal stability of bovine immunoglobulins during processing - Kinetic considerations. ICEF 12 - International Congress on Engineering and Foods, Québec, Canada, June 14-18

13 The presenting author(s) is (are) underlined.

Dumpler J, Kulozik U (2015) Reaction kinetics of heat-induced aggregation in skim milk concentrates - Comparison of lab scale indirect heating and direct steam injection. 9th NIZO Dairy Conference, Papendal, The Netherlands, September 30-October 2

Heidebrecht HJ, Hartinger M, Dumpler J, Kulozik U (2015) Milk protein fractionation with spiral-wound membranes to obtain native protein fractions. IDF World Dairy Summit, Vilnius, Lithuania, September 20-24