Woodhead Publishing Series in Food Science, Technology and Nutrition

Animal feed contamination

Effects on livestock and food safety

Edited by Johanna Fink-Gremmels

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Animal feed contamination

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Preface

It is a pleasure to introduce on behalf of all authors *Animal Feed Contamination: Effects on livestock and food safety*, a handbook devoted to animal feed contamination. As indicated in the subtitle, feed contamination with microbiological or toxicological agents can affect animal health, productivity and welfare, but at the same time, feed safety is an essential prerequisite for the production of safe and wholesome food products from animals fit for human consumption. The sentences above indicate the diversity of the disciplines that are involved in animal feed production and the assessment of animal feed safety. However, even a handbook of this size can only incompletely address all aspects of feed contamination, as the chemical substances occurring in forages and plants used in feed production with nutritional and anti-nutritional or toxic properties are virtually innumerable. Likewise there are many opportunities for microbiological deterioration and/or contamination with animal pathogens and potentially zoonotic agents. Hence this handbook aims merely to provide an introduction to the complex matter of animal feed contamination, highlighting a number of subjects of recent concern, as well as illustrating the impacts of harmonization of legislation and strategies put in place by the global feed industry on feed quality and safety.

The main areas addressed in the handbook include **microbiological hazards**, contamination of feed materials by **persistent organic pollutants and toxic metals** and the risks associated with the presence of **natural toxins**. On a global level, antimicrobial resistance is considered to be one of the major threats to the successful therapeutic use of antibiotics to combat human and animal bacterial infections. This is one of the topics treated in the section devoted to **veterinary medical products** as additives or

contaminants in feed materials. An **emerging technology** linked to feed production is **genetic modification of crops**, as GM crops are an important source of the plant biomass used as feed material. Their safety for the animal, the consumer of animal-derived products, and diverse ecosystems is a matter of controversial debate. Other emerging technologies generate new challenges for those responsible for feed safety, for example in the case of the production of **biofuel** from plant materials, providing guidance on safe levels for the use of the by-products of biofuel production in feeds. A short introduction to the potential risks associated with nanoscale feed ingredients is also presented.

The final part of the handbook is devoted to technical aspects of **Quality Management**. Authorities in all continents have set legal standards to guarantee feed safety for the animal as well as safe food supplies for the consumers of animal-derived products. Safety assessment is increasingly based on a stratified procedure, including all four components of **risk assessment** such as hazard identification and characterization, exposure assessment and risk characterization. The feed industry is requested to endorse these strategies and comply with the legal provisions, which include **proper sampling techniques** and analytical controls. To this end the industry has established technical protocols (HACCP and GMP) for sourcing, tracing, processing and transportation and the related quality standards for feed materials and compound feeds.

Every author or group of authors presents relevant information from an individual angle, from his or her professional experience, and from the scientific literature available. This has led to diversity in the outlines of the individual chapters, and diversity is also inevitable given the inherent complexity of the topic under consideration. However, we do hope that this handbook is considered a useful introduction to the multifaceted area of animal feed safety and that it provides relevant information about quality control and intervention strategies.

J. Fink-Gremmels

1

Introduction to animal feed contamination

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Abstract: Global acquisition of feed materials has become common in order to achieve economically viable production of animal feeds. At the same time this globalization increases the risk for potential microbiological and toxicological contaminants that determine the quality and nutritional value of feeds, but more importantly also the safety of animal-derived foods such as milk, meat and eggs. Quality control programmes require a stratified multidisciplinary approach addressing the entire food chain, from agricultural sourcing through processing to animal nutrition and assessment of animal and human health risks.

Key words: food and feed safety, concentrated animal feed operations, microbiological and chemical hazards, socio-economic impact of feed production, risk management and communication.

1.1 Animal feed production

Animal feed production is a silent industry that only reaches newspaper headlines at times of crisis; these crises include, for example, the emergence of unknown pathogens in livestock, such as prions (causing transmissible bovine encephalitis) (Sakudo *et al.*, 2011), unexpected sources of dioxin contamination of milk, meat and eggs (Hoogenboom *et al.*, 2010; O'Donovan *et al.*, 2011) and the presence of residues or unauthorized substances such as melamine in milk, milk products and animal feeds (Qin *et al.*, 2010).

The animal feed industry, however, is a truly global business of great economic importance. It is linked to concentrated animal feeding operations, with ever- increasing farm sizes trying to meet the increasing protein demands of the growing world population. Intensified animal production required the development of new principles of animal breeding, animal nutrition and feed composition (Thorne, 2007). The most impressive example of these changes is probably that of the broiler chicken, whose body weight at hatching is increased by up to 5000 times in the short 35

days of a broiler's lifespan. Diets for monogastric animals such as pigs and chickens may contain up to 60% cereal grains to achieve high productivity. This implies that in this case animal nutrition is in competition with resources that can be also used for human nutrition (Weckwerth, 2011). The grazing of cattle, originally widespread, has been substituted by dairy farms at which the cows are given an individualized diet containing up to 80% of concentrates, mixed according to the availability of energy-rich feed materials on the world market. The growing production of bioethanol/biodiesel adds to this division of resources and has resulted in a dramatic increase in the world market price of many feed materials such as soybeans, cereal grains and oil plants (Robertson and Swinton, 2005). The socio-economic impact of this competition is contributing to the global debate on the prudent use of the available natural resources and farmland and the prevention of land grabbing by strong economies such as Europe, where the net imports totalled more than 38 million ha in 2009 and 2010 (www.farmlandgrab.org; United Nations, 2010).

Considering these recent developments it is clear that the major concern in feed production is the availability of sufficient supplies of feed materials. As these are becoming increasingly limited, animal nutritionists have to meet the challenge of exploring the special dietary needs of high-producing animals while still ensuring that feed supplies remain safe and cost-effective. The high demands for an optimal feed utilization rely not only on diets able to provide the animal with all essential macro- and micro-nutrients but also require feed materials free of anti-nutritive factors and microbiological or toxicological hazards. Subsequently, feed safety has emerged as one of the most important parameters affecting animal husbandry, health and productivity.

1.2 Feed safety

1.2.1 From farm to fork

At the beginning of this millennium the European Commission presented its White Paper on Food Safety (EC, 2000; COM/99/0719 final) which was intended to improve transparency and safety along the entire food production chain. The term *from farm to fork* signalled the responsibilities of all stakeholders in the production chain to take effective measures to minimize risks to both animal and human health (www.ec.europa.eu/food/omtro_en.htm). Any food that reaches the consumer should be free from hazards such as microbiological or chemical (abiotic) contaminants. For both microbiological and toxicological hazards, the responsible international authorities (FAO/WHO and the Codex Alimentarius Commission (CAC), the US Food and Drug Administration (FDA), the US Environmental Health Protection Agency (EPA) and the European Food Safety Authority (EFSA)) present risk assessments and establish maximum tolerable limits for

contaminants, which serve as the reference for quality assurance programmes. In the initial phase of the production of foods from animal origin, feed quality is the most crucial factor that can lead to exposure of animals to undesirable contaminants. Therefore, within the European feed legislation it is stated that 'products intended for animal feed must be sound, genuine and of merchantable quality and therefore when correctly used must not represent any danger to human health, animal health or to the environment or adversely affect livestock production' (Commission Directive 2002/32/EC).

The feed industry has endorsed this need for transparency in the sourcing and processing of feed materials and in the use of feed supplements and additives. The latter require premarketing approval by the competent authorities prior to their use in feeds for farmed animals, including fish. Embedded in the One-Health Concept, integrated quality control programmes along the entire production chain should reduce the risk for animals and humans. It should also be mentioned that quality controls at the start of a production chain are a prerequisite for an economically viable agro-industry, thus preventing the need for rejection and destruction of food that is considered unfit for human consumption.

1.2.2 A truly multidisciplinary task

The assessment of feed safety is an extremely complex issue that has long been underestimated. Feed safety assessment in fact requires expertise in multiple disciplines such as agriculture and crop production, feed processing and technology and animal nutrition. Added to these basic disciplines, feed safety assessment also requires an understanding of microbiology and biosecurity measures, toxicology and animal health sciences (veterinary medicine) and ultimately experience in risk assessment methodologies.

Risk assessment has evolved into a well-structured scientific approach, with transparent rules, extensive data sourcing and distinct statistical procedures. The four essential elements of a quantitative risk assessment are hazard identification and characterization, exposure assessment and risk characterization. The ultimate outputs of risk assessment procedures are health-based guidance levels expressed as acceptable daily intake and tolerable weekly intake levels that carry no or a negligible risk for human health (Dorne, 2010). This stratified procedure must also be implemented in the assessment of feed safety for feeds used in food-producing animals. In addition, public interest has today extended to include the impact of large-scale animal production and feed sourcing on the environment and the role of farmers as eco-agricultural stewards (Thorne, 2007; Sachs, 2010).

1.2.3 Animal health and welfare

As previously mentioned, feed material may be the source of microbiological and chemical hazards (Frazzoli and Mantovani, 2010). Technical

processes such as cleaning, disinfection and heating are generally employed to damage and destroy microbiological contaminants that could otherwise cause contamination of food derived from these animals. These processes are cost-intensive and the efficacy of preventive measures needs to be established and controlled for any feed material and compound in feed. Hence the limitations are obvious: highly process-resistant agents such as prions (TSE-BSE) require inactivation processes that compromise the palatability and nutritional value of feed materials (animal by-products) (Sakudo *et al.*, 2011). The innate resistance of many bacteria (anaerobic, spore-forming organisms) to inactivation procedures such as heat and acid treatment leads to an ever-present risk of viable pathogens being present in individual feed batches, which are then introduced into animal facilities. Perhaps the most prominent example of an unresolved, long-lasting problem in feed hygiene is contamination with enterobacteriaceae such as *Salmonella*, *Campylobacter* and anaerobes (*Clostridium* spp.) in poultry units (Mataragas *et al.*, 2008; Fosse *et al.*, 2009).

Problems relating to toxic and anti-nutritive chemical substances are on an even wider scale. The EC European Catalogue of Feed Materials currently covers more than 700 entries, and all of these feed components may be potentially contaminated with one or more toxic substance(s). In the past, interest focused particularly on environmental pollutants and contaminants such as persistent organic pollutants (POPs), present in a broad variety of feed materials and able to accumulate in animal tissues. This is also true of dioxins and dioxin-like polychlorinated biphenyls and of many other polyhalogenated chemicals that are used in industrial processes and hence reach the environment (Fries, 1995; Antignac *et al.*, 2006; Yu *et al.*, 2010). Toxic heavy metals (cadmium, lead and mercury) and other chemical elements (arsenic, chromium, copper and zinc) occur naturally, but industrial processes may increase their concentration in distinct geographic regions, leading to their accumulation in plants that are consumed or harvested as feed for animal consumption (Schauder *et al.*, 2010; Lopes *et al.*, 2011). Moreover, recently identified new endpoints of toxicity, such as for example developmental neurotoxicity of methyl-mercury, gain increasing attention (Dorne *et al.*, 2011; Farina *et al.*, 2011).

Natural toxins such as toxic plant metabolites (i.e. glycosinolates, saponins, alkaloids, including pyrrolizidin alkaloids, and terpenes) and bacterial and fungal toxins (mycotoxins) have long been known to the veterinary professionals as causes of acute intoxications in individual animals, but have been largely ignored in risk assessment exercises. Their antinutritive properties and toxicological profile as substances with immunosuppressive and/or reprotoxic effects, however, have placed natural toxins high on the priority list for current risk assessment and statutory limits to protect animal health and performance. The risk assessment of such natural toxins is characterized by a high level of uncertainty as outlined in detail in the Opinions of the EFSA Panel on Contaminants in the Food Chain

(http://www.efsa.europa.eu/en/panels/contam.htm). Hazard identification of natural toxins is often based on simple case reports describing the adverse effects of a plant or plant material on animal health. These reports tend to lack a clear indication of the toxin concentration and the amount consumed. However, in order to accurately characterize this type of hazard, the dose–effect relationship needs to be established. Moreover, adverse effects observed after consumption of feed materials by the animal could be associated with one or more plant secondary metabolites (PSMs) and the number of possible mixtures of undesirable substances multiplies in complex feed mixtures and animal diets (Fink-Gremmels, 2010). Remarkable progress has been made in methods for analysing the complex pattern of natural toxins occurring in animal feeds, but the technical challenge still remains, as specialized technical knowledge in modern (multi-toxin) analyses is required.

An even larger margin of uncertainty must be taken into consideration in exposure assessment. Plant metabolites and fungal metabolites have a biological function in the natural ecosystem. They may offer the plant or fungus protection against insect damage or pathogen invasion (antiviral and antimicrobial properties) or improve resistance to environmental stress factors (heat, drought, excessive rainfall, nutrient shortage). Inherent to these biological functions, the amounts of synthesized toxic metabolites vary significantly, resulting in a broad (unpredictable) range of actual feed contamination levels (Orians *et al.*, 2011). Hence exposure assessment must take this broad concentration range into account and match this with dietary requirements or common local feeding practices. The composition of a diet, however, varies widely across different geographic regions and correlates with farming practice. Even for one animal species feed composition changes continuously according to age and production status (MacLachlan, 2009). A conservative (precautionary) approach in the assessment of chemical substances in animal feed material will result in a shortage of commercially available feed, while a more generous approach means that the risk remains of adverse effects on the health, welfare and productivity of the animal and of the formation of residues in edible tissues, milk and eggs. The level of uncertainty in exposure assessment may be reduced through the collation of large sets of data on the occurrence of chemical feed contaminants and a detailed assessment of animal feed consumption patterns. Within Europe, this strategy has recently been implemented by the EFSA, who have based animal exposure assessment on large data sets established from thousands of feed analyses conducted in the different Member States during the routine control of feed materials or within the framework of national or Europe-wide monitoring programmes. This approach seems to be very cost-intensive, but still outweighs the high costs of recalling contaminated food batches from the market and, more importantly, reduces the actual risk of accidental human exposure.

1.2.4 Human hazard identification and characterization

Food from animal origin contributes to the exposure of human consumers to microbiological and chemical hazards. *Microbiological hazards* mostly comprise (zoonotic) bacteria and their endo- and exotoxins that are incompletely inactivated during food processing. Viruses are generally (animal) host-specific: food contamination with viral agents is less prevalent and can usually be attributed to secondary contamination during food processing and handling. Exceptional pathogens are prions, which have emerged as contaminants of feed and probably food with high process stability. The most prevalent food-borne diseases, however, can still be attributed to the classic pathogens such as *Salmonella*, *Campylobacter*, *E. coli*, *Listeria* and in some cases *Clostridium* species, as mentioned above. Insufficient hygienic barriers both at farm level as well as in food processing and handling account for this risk (Doyle and Erickson, 2012). One of the corrective actions employed at farm level was (and still remains) the use of antibiotics to counteract clinical and subclinical infections in animals. One well-received side-effect of the use of antibiotics is their growth-promoting effect in target animal species. The abundant use of antibiotics in livestock (including fish) seems to contribute, however, to the global emergence of antimicrobial resistance (Gilchrist *et al.*, 2007). The horizontal transfer of resistant bacteria from living animals to humans has been well documented for MRSA (Methicillin-Resistant *Staphylococcus aureus*), involving all animal species so far investigated, including pigs, cattle, horses and domestic pets. Although the pathogenicity of the typical livestock MRSA (ST398) seems to be low in humans, there are concerns about a possible gene transfer between these less pathogenic strains and the highly pathogenic hospital-acquired MRSA strains (Graveland *et al.*, 2011; Garcia-Graells *et al.*, 2011). The same applies to *E. coli* isolates expressing Extended Spectrum β-Lactamases (ESBLs) (Liebana *et al.*, 2006). As the genes encoding for ESBL are located in mobile transmissible elements (plasmids), gene transfer between animal-born isolates and human *E. coli* strains cannot be excluded. An increasing prevalence of pathogens that are resistant to multiple and perhaps in the future to all known classes of antibiotics is currently considered to be the most serious hazard for the human population.

In contrast to the microbiological hazards associated with handling and consumption of animal-derived foods, the prevalence of *chemical hazards* such as residues in edible products is much lower, despite the increasing concerns about long-term subclinical effects (Miraglia *et al.*, 2009). The animal has the function of a filter in which the toxin burden of chemical feed contaminants is generally reduced as a result of biotransformation and elimination by the animal. However, there are well-known exceptions to this general paradigm. Some persistent pollutants accumulate in the animal, such as heavy metals in the kidneys of herbivores, and organic pollutants in fatty tissues including fish meat. Special attention is paid to the excretion

of potentially toxic substances in milk, as infants consume relatively high amounts of milk when consumption is corrected by the (low) body weight and exhibit an increased vulnerability to developmental toxicity such as neurotoxicity and immune toxicity (Miodovnik, 2011). Due to these various mechanisms the low prevalence and small concentrations of chemical residues create a very limited human exposure to chemical contaminants of milk, meat and eggs. Consumer perception, however, differs significantly from this scientific evaluation.

1.3 Risk management and communication

The competent authorities are responsible for translating the results of scientific risk assessment into enforceable and controllable legislation, aiming at optimal consumer protection. This has resulted in a broad framework of legal provision and analytical controls for chemical contaminants, and testing for compliance with statutory limits. The most recent compilation of the data from chemical residue monitoring in pig meats was conducted by EFSA (published in October 2011; http://www.efsa.europa.eu/en/efsajournal/pub/2351.htm), indicating a prevalence of non-compliant samples of less than 0.34% for all chemicals (including veterinary drugs) with the highest level of 1.26% of non-compliant samples recorded for chemical elements (heavy metals and metalloids). The total sample size included more than 800,000 analytical results provided by the national residue monitoring programmes conducted in the EU Member States. It should be reiterated that the given percentages refer to the fact that a tissue sample was non-compliant with the current legislation, which does not mean that the measured concentration constitutes a direct health hazard for the consumer of the product. Of this total number of positive samples, the actual number that exceeded statutory limits was even lower. Comparable data analyses for other animal species such as poultry, small and large ruminants and wildlife species are in progress. This evaluation of residue monitoring is of the utmost importance in identifying shortcomings in the control of the production chain from *farm to fork* and to set priorities for corrective measures. It is also of importance for risk communication, providing the consumer of animal products with the necessary information on the actual health risk. Although these results convincingly indicate that the human health risks are low, the contamination of feed with undesirable substances remains a risk for animal health and welfare. In the presence of toxic (undesirable) substances, the health status of the animal is known to decrease, thereby increasing the risk of infectious diseases. This may in turn increase the risk of products from diseased animals, which contain microbiological contaminants, entering the food chain, and also contributes to an increased use of antimicrobials (antibiotics). This in turn may contribute to the global emergence of antibiotic-resistant pathogens. It remains essential

to communicate these different risk scenarios to risk management, stakeholders and consumers (Thorne, 2007).

1.4 Future trends

As a result of changes in agricultural practice and of the availability of feed materials that include more and more secondary (by-)products of technical processes (bio-energy) and recycled products, in order to protect declining natural resources, stakeholders, animal nutritionists and animal health professionals and the competent authorities will need to devote attention to feed safety at all times. In addition, the global transport of feed materials offers numerous opportunities for the post-harvest contamination of feed materials with undesirable microbiological or chemical agents. The transport of feed materials may also serve as a vector for the trans-continental transfer of (resistant) pathogens that affect not only animal but also plant health, and contribute to the spread of invasive weeds and infective agents. Global changes, such as climate change, will require production sites for feed materials to be reallocated, a measure that may prove to be of great socio-economic impact. It is likely that animal operations will fall into two entirely different sectors: on the one hand, conventional and sustainable rural (organic) farming with a low input of external feed resources, and on the other, large-scale animal operations that are needed to cover the increasing need of the world population for animal proteins. In turn, feed safety assessment has to acknowledge these different production systems and provide tailor-made solutions to enable effective integrated quality programmes that protect animal and consumer health.

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2

Animal feeds, feeding practices and opportunities for feed contamination: an introduction

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Abstract: Feeds for farmed livestock must satisfy the requirements of both the animals and the ultimate consumers of animal products, particularly in respect of safety. Feed legislation provides comprehensive safeguards but incidents that threaten feed and food safety still occur. The grazing animal may be faced with a number of hazards brought about by the geology of the area or industrial activity, or by plants that contain natural toxins or become infected with microbial pathogens. Harvested feeds may be contaminated in store, during processing, mixing or transportation as a result of spoilage or contact with unapproved feeds and non-agricultural materials being carried in the same vessel.

Key words: feeds, feeding, contamination opportunities, rapid alerts.

2.1 Introduction

Feeds are primarily aimed at satisfying an animal's nutritional needs for maintenance, activity, production and reproduction. However, farmed livestock are reared to produce meat, milk and eggs for human consumption and feeds for such animals must also satisfy the requirements of the ultimate consumers of all products of animal origin. Nowhere is this more important than in the consideration of food safety and it is apposite that the protection of human health is a fundamental objective of the European Food Law (EC, 2002b), with feed destined for food-producing animals considered within that context. In other words, animal feed is recognised as being part of the human food chain, and any consideration of feed safety needs to include both the risks for the animals that eat it and those that may affect the human consumer of animal products.

2.1.1 Feed legislation

Animal feed is defined rather broadly in European legislation, but there is an overriding stipulation that feeds placed on the market should be safe, sound, genuine and fit for purpose and have no adverse effect on animal welfare or the environment (EC, 2009a). Such non-specific requirements are supported by quantitative controls on the presence of undesirable substances in feed, such as toxic metals (e.g. arsenic and cadmium), persistent pollutants such as dioxins or natural toxins (EC, 2002a), and a number of materials, including treated seed, tanning wastes, faeces and waste waters that are specifically prohibited. These additional proscriptions help to complete a comprehensive safeguard against known hazards in feeds. The scope of the undesirable substances legislation was extended by the Feed Hygiene Regulation (EC, 2005) to ensure safety throughout the feed chain by imposing measures to control spoilage and contamination from crop and feed production, through storage, transport and processing to the eventual feeding of the material. It has been estimated by the EC Commission that, in 2007, some six million feed business operators fell within the scope of this Regulation (EC, 2007).

2.1.2 Feed assurance

Running alongside these legal controls, the feed industry in various countries has developed voluntary codes of practice, e.g. GMP Plus in the Netherlands, OVOCOM in Belgium, and AIC Assurance Schemes (2009a–c) in the UK. They aim to ensure safe practices in the production, processing, storage and transport of feeds, and they demand that assured farmers buy feed only from assured suppliers, who in turn purchase their raw materials only from assured producers. Independent audits ensure that each link in the chain complies with the required standard so that, collectively, these assurance schemes ensure safe food and help to build consumer confidence in the safety of the feed chain that supports the production of food products from livestock farms.

It seems self-evident that assurance schemes should be interlinked in order to provide comprehensive control throughout the feed chain, but each link has been built step by step as new threats have been identified, assessed and controlled. Formal risk assessments based on HACCP principles (Codex 2003) are an integral part of the codes of practice, and they later became a legal requirement in the EU when the Feed Hygiene Regulation of 2005 was implemented for all businesses that had any involvement with animal feed supply.

The potential risks posed by an incomplete safety chain may be envisaged in situations where there is widespread contamination of the growing crop, such as by aflatoxin in maize, and an accompanying feed shortage. Feed chain assurance should make certain that contaminated supplies are

identified and that none is included in feed products that will be incorporated in the animals' diet.

2.1.3 Some recent contamination incidents and their costs

Even with the well-developed feed protection controls that have been established in Europe, incidents that threaten food safety continue to occur and complacency would be misplaced. In a summary of 'things that went wrong', Millar (FSA 2009) listed 26 feed contamination incidents that had an impact on the European food and feed markets between 1998 and 2008 – beginning with dioxins in Brazilian citrus pulp and ending with the same toxic chemicals found in processed bread in Ireland. A summary of those contamination incidents is given in Table 2.1. Since that report another major incident of dioxin contamination of feed fat occurred in Germany in late 2010.

The financial cost of such incidents can be very high and Table 2.2 gives the EC Commission's own estimates of some major incidents. However, it is difficult to make a full assessment of costs and, as the BSE disaster has shown, there may be long-term implications beyond the point at which the initial problem has been brought under control. In the past, much of the cost has fallen on the public purse, but that may not continue to be the case in future. Although a system of financial guarantees has yet to be agreed, the Feed Hygiene Regulation (EC, 2005) did establish the principle that feed business operators should be liable for the cost of feed safety infringements.

2.2 Feeds and feeding practices

Recent statistics for the European Community of 27 Member States indicate that the total amount of livestock feed consumed is of the order of 460 million tonnes per year, expressed on a fresh weight basis (FEFAC 2010). Almost half of this total comprises forages that are mostly grown and consumed by ruminant livestock on the farm of origin. The other, non-forage, half of the European livestock diet is consumed by both ruminants and non-ruminants and only 25% of that is grown on the home farm, with almost as much again imported from countries all around the world.

2.2.1 Forages

Fresh herbage constitutes the major part, if not the whole, of the diet for many sheep and herds of suckler cattle throughout the growing season. Its seasonal growth pattern permits surpluses to be cut and conserved as silage or hay for feeding when herbage growth is restricted by temperature or drought, and typically these would be stored and used as the main feed

Table 2.1 A decade of contamination incidents

Contaminant	Year	Feed	Origin
Dioxins	1998, 1999, 2000, 2002, 2003, 2004, 2006, 2008	Citrus pulp, feed fat, compound feed, copper and magnesium supplements, bread meal, dried grass, potato co-product	Brazil, Belgium, China, Germany, Netherlands, Spain, Ireland
Antibiotic	2002	Milk powder	Russia, Ukraine, Belarus
Pesticide	2002	Wheatfeed	Germany
Hormone	2002	Glucose syrup	Ireland
Hydrocyanic acid	2003	Linseed pellets	USA
Animal protein/ bone	2004, 2006	Sugarbeet pulp, maize co-products, biscuit meal	EU, USA
Unauthorised GMO	2005, 2006	Maize gluten feed and rice	USA, China
Unauthorised organic selenium	2005	Yeast product	USA
Cadmium	2006	Zinc sulphate	China
Melamine	2007, 2008	Pet food, organic soya expeller	USA, China
Salmonella	2008	Wheat protein	UK
Aflatoxins	2008	Sunflower seeds	Egypt

Source: FSA (2009).

Table 2.2 Financial cost of some major feed contamination incidents

Country most affected	Year	Cost (€ million)	Incident/ contaminant	Contaminated feed
UK	1986	2750[1]	BSE	Meat and bone meal
Belgium	1999	181[2]	Dioxins	Fat
Germany	2002	1[3]	Nitrofen	Cereals
Netherlands	2002	43[4]	MPA	Glucose syrup
Germany	2003	0.7[5]	Dioxins	Dried bakery products

[1] Recurring annual cost of BSE (bovine spongiform encephalopathy).
[2] Direct costs in Belgium only.
[3] Direct costs in the two most affected German Länder (nitrofen is a banned pesticide).
[4] Direct costs in the Netherlands only (MPA is a hormone product, *viz*. medroxyprogesterone acetate).
[5] Direct costs in Germany only.
Source: Details extracted from EC (2007).

source on the same farm. Other forages, such as root crops, kale and forage rape, may be grown to help fill gaps in the herbage supply, and they would typically be grazed *in situ*. Forage maize may be cut and fed green in late summer before the rest of the crop is harvested and ensiled ready for use at other times of year. Most dairy herds and fattening beef cattle, other than

feedlot cattle, also consume fresh or conserved herbage as a substantial component of their diet, though such productive animals are commonly given additional nutrient-dense supplements.

Both hay and silage making have long histories: Schukking (1976) considered haymaking to be the oldest method of herbage conservation, although he noted that silage making was carried out some 3000 years ago. During and after the 1970s the advent of large, powerful equipment, capable of handling heavy crops of wet grass, triggered a major switch from hay to silage making on large farms. The change was also influenced by considerations of weather dependence – whereas well-preserved silage could be conserved with minimal delay between cutting and collection, hay required a minimum of three to four days' field wilting to achieve the required dry matter content. Additives have been introduced into both practices in order to enhance preservation, but they have been used much more widely in silage making where their efficacy has been improved by the development of applicators that achieve better incorporation into the crop. Silage preservation was also much enhanced by improvements in the silo – solid floors, airtight walls and the liberal use of a polythene seal. However, even if low oxygen conditions could be achieved and maintained in a well-sealed silo, the silage may still be vulnerable to decay when the silo is opened. Such silos may hold several hundred tonnes of silage, and be expected to feed a herd for several months, but such a prolonged system of feeding provides a significant opportunity for oxygen to penetrate some distance behind the open face and create conditions where aerobic spoilage could occur. Paradoxically, the solution to this problem for some silage makers was to go back to a smaller scale; to eschew the silo completely and conserve well-wilted grass in large round bales (300–400 kg each) that were sealed individually by multiple wrapping with plastic film. In this system some additional field loss is compensated by substantially reduced aerobic degradation since the bales are opened and fed on the same day. Maize, despite some propensity to aerobic decay, is rarely, if ever, conserved as baled silage and this is a fertile situation for the use of effective silage additives.

2.2.2 Compound feeds

More than 60% of the non-forage part of the diet is fed in the form of compound feeds. Some are marketed as a loose mixture in which individual ingredients may be identified, but most are extruded into pellets of various sizes suitable for different classes of animal and into 'crumbs' for some poultry feeds. The feed groups that make up the compound feed fraction are quantified in Table 2.3.

More details of individual feed ingredients are contained in the EU Catalogue of feed materials (EC, 2010), which includes 166 entries in 12 categories, but the EU has resisted proposals to produce a comprehensive list of all the feeds used within its borders. Such a list would have had the potential to extend, like its American equivalent (AAFCO), to many

Table 2.3 Feed materials in compound feeds (thousands of tonnes per annum)

Cereals	71480
Oil cakes and meals	40829
Food co-products	17139
Oils and fats	2187
Dried forage	1995
Pulses	1793
Dairy products	1149
Manioc	758
Animal meals	576
Minerals and vitamins	4285
Others	6034
Total	148225

Source: FEFAC statistics for the EU (2010).

thousands of entries and to require constant updating. However, the First Revision of the European Catalogue, prepared by a Feed Chain Task Force (representing producers, purchasers, traders and users of feed materials), was implemented in 2011 and this has a total of 568 entries. An additional Register of feed materials has been introduced for feeds not listed in the Catalogue and this is open-ended.

Cereal feeds

The FEFAC data confirm that cereals and cereal-derived feeds are the major components of European compound feeds. This group includes a wide range of cereal feeds from whole grain to fractions that have been subjected to or separated by various processes such as rolling, grinding, flour milling, starch extraction and both dry and moist heat treatments. An indication of the variety of cereal feeds can be seen in the Revised EU Catalogue where 120 cereal feeds are derived from barley, maize, millet, oats, rice, rye, sorghum, spelt, triticale and wheat. Other than whole grain, the most widely used cereal feeds would be wheat middlings and bran, which comprise the outer layers of the grain that are removed during milling in the production of various grades of flour. Occasionally the flour itself also becomes available for use as feed.

Oil cakes and meals

Oil cakes and meals make up 28% of compound feeds, and this is a crucially important fraction since it provides much of the dietary protein. This group of feeds derives from the seeds or fruits of plants such as cocoa, coconut palm (*Cocos* spp.), cotton, groundnut, linseed, niger, olive, palm kernel (*Elaeis* spp.), rapeseed, safflower, sesame, soyabean and sunflower. The raw materials are rich in oils that are valuable food products in their own right, and the oil is typically expressed or solvent extracted (either in Europe or

in the country of origin), which leaves the protein-rich residue that is used as animal feed. The EU grows only a small proportion of the oil-rich crops – mainly rapeseed and sunflower – from which its protein-rich feeds are derived. Currently the EU's self-sufficiency ratio in relation to its feed protein needs is only 23%, and Europe is particularly deficient in soyabean meal, a high-quality protein source that is widely used in pig and poultry formulations.

Food co-products and surplus foods

Food co-products represent fractions separated by food processors in their effort to meet a wide variety of product specifications. Sugarbeet pulp is separated from the sugar-rich liquor by hot water extraction of small pieces of sugar beet and is widely used as a palatable and versatile ruminant feed, as is the molasses that remains after sugar refining. Citrus pulp is another feed material produced when citrus fruit – principally oranges – are pressed to release their juice, and citrus molasses is also produced. Such materials demonstrate the synergy of the food and feed industries, though it sometimes needs to be emphasised that co-product feeds are not derived from sub-standard raw materials or from food industry wastes; rather they are fractions of food quality materials that humans choose not to eat (Crawshaw 2001).

This feed group also includes surplus foods such as bread, cakes, biscuits and confectionery, which differ from co-products in that they comprise the whole food product, that is a processed mixture of ingredients rather than a single raw material or separated fraction. Surplus foods often arise in a bakery or food factory, where they are genuinely surplus or they fail to meet strict product specifications, and some may be returned from retail outlets as unsold.

Oils and fats

Oils and fats largely derive from the oilseeds and fruits referred to in the section on oil cakes and meals and, since both fractions are used as feed materials, this may stimulate the question of why they were separated in the first place. The answer is that the separated oil is widely used for a number of purposes other than feed, and the protein-rich fraction – which may also have other non-feed uses – may be fed to livestock at higher dietary levels when it is not associated with the high oil content that is present in the raw material. However, that is not to say that whole oilseeds have no utility as animal feeds and 'full-fat soya', for example, is used for both ruminants and non-ruminants where a high-energy formulation is required (Monari 1994).

Dried forage

Dried forage is produced by the artificial drying of crops, principally grass or lucerne, and is commonly ground and pelleted after drying. Multiple cuts

are often taken at set intervals from fields within a reasonable distance of the drying facility and, in order to meet the demand for feed transparency, leading operators are now able to link the product to the field on which it was grown (BAGCD, n.d.). Rapid drying largely precludes the loss of quality in the field, and the high temperatures employed may enhance protein quality for ruminant stock. The product is commonly used as a source of fibre and sometimes of β-carotene.

Pulses

Pulses have been encouraged by EU subsidies because they represent protein-rich feeds that can be grown in Europe. Most commonly grown are peas and beans and there are some processed pea co-products, such as pea middlings and bran, that are also used as feed. Other pulses included in the European animal diet include chickpeas, lentils, sweet lupins and vetches.

Dairy products

Dairy products in feed are largely confined to diets for young, unweaned animals. They are produced in milk processing plants and may derive from fat-separated milk or from a number of other processes such as butter churning and cheese manufacture, or the production of casein and lactose. All dairy products used in compound feeds are dried to a notably low moisture content of 5–8%.

Manioc

Manioc is produced from dried cassava roots grown principally in Thailand and Indonesia. The material often has a floury nature and, for this reason, it is commonly used in pellet form. Manioc is rich in starch, which is sometimes extracted, and both the starch and the extracted residue are used as feed.

Fish and land animal meals

In former times, fish and land animal meals were valued as high-quality protein sources and used in a range of diets where protein quality was of particular importance. Usage has fallen as a direct consequence of the BSE problem (which was attributed to contaminated animal meals) and concern about the sustainability of fish stocks. At present, any meal made from mammalian tissue is banned from mammalian diets within the EU, and thus usage is largely restricted to poultry feeds. Fish meals are also banned from ruminant diets in the EU and their greatest usage is now in the fish feed sector.

Minerals

Minerals are included in animal feeds in a range of organic and inorganic compounds to supply specific mineral elements. Thus calcium, for instance, is provided as calcium carbonate (from limestone deposits, crushed oyster or mussel shells and calcareous marine algae), dicalcium phosphate, calcium

chloride and others. Sodium is included as sodium chloride, sodium bicarbonate, the sodium salts of fatty acids and others. Some materials are described as 'technically pure', implying a degree of chemical processing, while others are 'products of natural origin', materials that are closer in composition to the original rock or marine sources. Mineral elements can be divided into two groups:

1. Those needed in relatively large amounts – the mineral elements calcium, phosphorus, magnesium, sodium, potassium and chlorine – and minerals supplying these elements are classified in the EU as feed materials.
2. Those needed in much smaller amounts – such as iron, manganese, copper, zinc, cobalt, iodine and selenium – and minerals supplying such 'micro-elements' are regarded as feed additives. Note that all vitamin supplements are also categorised in the EU as additives.

The distinction between feed materials and additives is significant, for the latter group is subject to strict controls in European legislation (EC, 2003). Before being placed on the market, additives are required to undergo safety assessment and to achieve official authorisation, and their use may be restricted to specific purposes for specific animal species.

Other feed additives

In addition to the supply of micro-nutrients that are essential to the nutrition of farm animals, other feed additives are included in compound feeds for a variety of non-nutritional or additional purposes (Annex to EC, 2009b). Some act as preservatives or binders, others add flavour to the diet or colour to egg yolks, while other additive groups influence the gastrointestinal flora or the viscosity of the digesta, reduce the ammonia content of the faeces or improve nutrient digestibility. A further group of feed additives includes coccidiostats and histomonostats, which have a prophylactic effect against protozoal infection. Antibiotic growth promoters were previously permitted as feed additives, but their use was banned after 2005 when it was concluded that they contributed to the development of resistant strains of pathogenic organisms and presented a potential threat to human and animal health (EC, 2003). Medicated feeds are provided only under veterinary prescription. They represent a supply route for medicines, though their use is strictly controlled to ensure the correct dosage is supplied to the correct animals and to avoid the appearance of residues in animal products that may enter the food chain.

2.2.3 Other dry feeds

The FEFAC data indicate that 84 million tonnes of non-forage feeds are fed to EU livestock in addition to those purchased as compound feeds. More than 60% of this total is made up of cereals that are grown and used

on the same farm. The remaining 30 million tonnes of other feeds include a number of feed materials that may be fed alone or blended into a home-mixed ration on-farm. Some of these feeds – such as sugarbeet pulp, maize gluten feed and oilseed meals – are identical to those used by feed compounders. Mineral mixtures are also used separately and may be added to a farm blend or offered 'free choice'.

2.2.4 Other moist and liquid feeds

A wide range of moist and liquid feeds may also be included in livestock diets, and such feeds appear to be underestimated in the figures given in Table 2.2. Moist feeds are fed throughout Europe, and the Netherlands Feed Producers' Group, OPNV, recently compiled data which show that, collectively, they amount to 35 million tonnes within the 27 Member States of the EU (OPNV, personal communication, 2009). Moist and liquid feeds are occasionally mixed and marketed as moist feed blends, but they are more typically supplied directly from the production site to the farms where they will be used. They derive from a range of agro-industrial activities such as brewing, juice extraction and potato processing and are separated in operations that involve the extraction or fractionation of cereals, fruit, roots and tubers.

Some food processing operations yield only a limited number of co-products, as in the brewing of beer where solid brewers' grains are separated from the liquid extract and brewers' yeast becomes available after this liquid has been fermented. In other situations there are a number of separate co-product streams, as in the frozen potato industry where processing may lead to the production of peel, uncooked off-cuts, cooked mash, out-of-spec fried products, some dried potato and feed-grade starch separated from the factory's internal water stream. In some forms of cereal processing the whole operation involves a fractionation of the raw material into identifiable parts that are supplied to both human food and animal feed markets (Crawshaw, 2001).

2.2.5 Feeds from bio-fuel production

The development of the bio-fuel industry in recent years, and its future expansion, is likely to result in a substantial increase in the supply of co-product feeds (Cottrill *et al*. 2007). Essentially there will be two types of operation to produce: (1) bio-diesel from tallow and vegetable oil-rich materials, and (2) bio-ethanol from cereals and possibly other starch or sugar-rich raw materials. The co-product feeds will initially resemble those produced during oil extraction for food purposes (oilseed cakes) or in potable spirit production (distillers' grains), though future developments may utilise other raw materials and these will be reflected in different co-product outputs.

2.3 Feeding systems

Above subsistence farming levels, non-ruminant livestock are typically fed harvested and processed feeds that can be controlled in terms of both quantity and quality. Some of these feeds may have been grown or manufactured locally, while others will have been transported thousands of miles by road, rail and water. Commercial units of free-range pigs and poultry may provide greater space for their livestock, but there is commonly little divergence from the diet that is fed to animals maintained indoors. Organically grown stock are fed different diets, in that they do not include genetically modified materials or feeds that have been produced with the aid of pesticides or inorganic fertilisers. However, like conventional feeds, organic feed materials are not all grown locally and they too may have been subjected to different climatic and storage conditions, and different legislative controls, from those at home.

Most ruminant stock spend at least part of their lives outdoors and graze grassland and forage crops that may have been grown for the purpose. However, in upland areas non-dairy sheep and goats may pass much of their lives outdoors and consume diets that owe much to nature and less to the design of man. In the upland situation animal choice may play a significant role in determining the daily diet; studies have shown that sheep and goats may select very different diets from the same hillside. And when grazing is in short supply, whether by virtue of drought or low temperature, the herbage that is consumed is inevitably accompanied by a proportion of soil. Where grazing is supplemented by other feeds, these may be confined to late pregnancy when increasing nutritional demands – particularly in animals carrying more than one foetus – coincide with a restricted appetite.

Suckler herds of beef cattle may enjoy the same freedoms and privations as hill sheep but, in the developed world, their diet is likely to be supplemented for longer periods by conserved forage and possibly other feeds. Such feeds compensate to some extent for the relative inability of cattle to graze as close to the ground as sheep, and the supplemented diet may support a higher level of production where this is required. Dairy herds (of cows, goats or sheep) need to be fed at a level that will sustain lactation. In pastoral countries, such additional energy and nutrient requirements may be met from the pasture. In New Zealand, for example, seasonal calving has traditionally been practised in an attempt to match the national dairy herd's peak nutritional demands to the seasonal growth of grass. In countries where year-round pasture growth does not occur, or where higher levels of individual performance are required, supplementary feeding is an entrenched part of the system. The diet of highly productive dairy cows often comprises a high proportion of nutrient-dense feeds with only a minimum proportion of herbage, forage or straw – long fibrous feeds provided to ensure satisfactory rumen function.

Non-ruminant animals have little or no ability to digest and absorb fibre and could not sustain an adequate level of production on forage diets. Pregnant sows can make some use of fibrous feeds owing to their large appetite, some caecal fermentation and relatively limited nutrient requirements, but other classes of pigs and poultry must be fed more nutrient-dense diets. They likewise have no ability to utilise non-protein nitrogen and pig and poultry diets must contain sufficient amounts of high-quality protein, from sources such as soyabean meal, to meet the animals' needs for specific amino acids. Laying hens have a substantial need for dietary calcium to ensure satisfactory shell formation, and suitable diets contain of the order of 10% calcium carbonate.

Horses can make some use of fibrous feeds but their ability is much poorer than that of ruminant animals. Since fibre digestion occurs in the caecum, beyond the main absorptive region of the digestive tract, the horse is unable to derive maximum benefit from such feeds. Thus horses required to grow quickly, or to sustain significant physical demands, must be given a diet containing a proportion of nutrient-dense or energy-rich feeds.

2.4 Feed contamination opportunities

The geological origin of some natural mineral sources may result in contamination by undesirable chemical elements as such as arsenic, cadmium, lead and mercury. The contamination of feeds of plant origin can occur in the field where they are grown – by weeds, microbial infection or environmental pollution – or at a later stage. Feeds of animal origin may carry the risk of transfer of pathogenic organisms from the farm or processing site to a new generation of livestock and, potentially, to the human consumers of animal products. Once harvested, processed or manufactured, all feeds are vulnerable to problems in storage, further processing, mixing and transport, and even to adulteration.

Water is not always considered to be a nutrient, though the EU Directive on undesirable substances (EC, 2002a) states that the same rules concerning the safety of animal feeds have to apply to the safety of water consumed by animals. The water supply is undoubtedly a potential route by which contaminants may enter the feed and food chain. Animals are often given no choice of water supply and are consequently unable to reject water in the way that they may reject individual feeds. Grazing animals may have additional access to waterways but this may expose them to toxic chemicals that are present as a result of contamination upstream, or they may be present in the run-off from neighbouring land. Private water supplies may become contaminated by effluents from farm slurries or from elsewhere. The elimination of such risks is not always possible, but consideration needs to be given to ways in which they may be reduced in order to safeguard animal and human health.

2.4.1 Environmental contamination

As noted above, the geology of certain areas leads to naturally high levels of potentially hazardous mineral elements in rocks and this can lead on to high levels in the associated soils, herbage and groundwater. Thus, since the 1850s the shale soils of South Dakota, Montana and Wyoming have been recognised as containing appreciable amounts of selenium and the herbage they supported was toxic to sheep and cattle. Similarly, the teart pastures of Somerset have long been known as an area where molybdenum-induced hypocupraemia may be expected in grazing animals (Ferguson *et al*. 1943). Potentially toxic levels of heavy metals may also occur as a result of industrial activity. Thus high levels of arsenic are found in groundwater flowing through rocks in which arsenic is a constituent of minerals such as iron pyrites, but arsenic is widespread in the environment today due to its use in a range of industrial operations including tanning, electronics and the production of insecticides, defoliants, pharmaceuticals and glass (Hindmarsh and McCurdy 1986).

As the finishing touches were being applied to this chapter in March 2011, the massive earthquake and resulting tsunami in Japan damaged the Fukushima nuclear power plant and raised the risk of radionuclide contamination of the feed and food chain in other parts of the world. Control measures on feed and food imports have been put in place in Europe, which require testing for the levels of iodine-131, caesium-134 and caesium-137.

2.4.2 Contamination in the field

Plants have evolved a variety of means of defending themselves against attack by micro-organisms, invertebrates and higher animals. They include the production of natural pesticides in the seeds and berries and in other parts of the plant. Their toxic effects are caused by a number of substances (including alkaloids, glycosides, other phytotoxins and volatile oils) which may occur singly or in combination, and their concentration varies with the soil, climate, altitude and season, as does their distribution within the plant (Forsyth 1968). Some plant toxins are extremely poisonous and ricin, found in the seeds of the castor bean (*Ricinus communis*), has been identified by Timbrell (2005) as the most toxic chemical known to man.

Animals have developed their own ways of overcoming or circumventing such challenges. Ruminant animals are more exposed to the risks presented by poisonous pasture plants because they are more reliant on pasture feeds and it is thus significant that the ruminants' digestive system provides them with additional protection. Rumen bacteria degrade a number of plant toxins to render them less harmful, and prior exposure to these substances increases the rate at which they are detoxified (Carlson and Breeze 1984). However, complete and comprehensive detoxification is not achieved in all cases, as is evidenced by the widespread incidence of bracken poisoning. This plant (*Pteridium* spp.) is found in every continent of the world,

including Antarctica, and is regarded as a potentially serious threat to the health of both animals and man (Taylor 1980). However, rumen fermentation is not always beneficial and, in other circumstances, rumen microbes play an unhelpful role by turning non-toxic substances into toxic derivatives. Thus nitrate is converted to nitrite and S-methyl sulphoxide is formed, both of which have caused toxic effects in animals grazing on kale (Carlson and Breeze 1984).

Domestic livestock will often avoid eating plants that they have found to be unpalatable or an irritant to the linings of the mouth, stomach and intestinal tract. It is thus common to see cattle safely grazing meadows that, from a distance, appear to be dominated by potentially harmful buttercups (*Ranunculus* spp.). Ragwort (*Senecio jacobaea*) is another highly toxic plant that grows widely in pastures – as well as on many areas of waste land – and, like the buttercup, it often remains ungrazed so long as there is an alternative supply of feed. However, there is a crucial difference between these two plants that renders buttercups relatively harmless while ragwort continues to be a major problem to both ruminants and horses. The toxic principle in the buttercup is an unstable chemical that is not found in hay made from such meadows. Thus, when offered the conserved crop, the animal's inability to selectively avoid the buttercup plants is of no significance. In stark contrast, the ragwort plant retains its toxicity at all stages and the presence of ragwort in conserved crops represents a considerable risk to stock that may consume it inadvertently. In his book on British poisonous plants Forsyth (1968) claimed that ragwort probably caused greater loss to the UK livestock industry than all the other poisonous plants put together. More than 30 years later Knottenbelt (2002–06) of the Liverpool University Veterinary School referred to ragwort as a growing threat to the UK horse population (*Equine Science Update 2002–2006*), which implies that the position has not improved.

The severity of plant poisoning depends on a number of factors that vary according to the plant as well as the species of grazing animal. These include the toxicity of the part that is eaten and the amount consumed as well as the age of the animal and its sensitivity. Sporadic problems sometimes occur as a result of farming practice. When bracken-infested land is ploughed up some of the roots may be left on the surface and present a greater risk of poisoning than other parts of the plant. This observation may be explained by seasonal variations within the plant, but bracken does present a number of health hazards to grazing stock and both the rhizomes and the fronds have been found to contain thiaminase (Evans *et al*. 1951), which may cause the condition known as bracken staggers in horses, and to be rich in the toxic aplastic anaemia factor (Evans *et al*. 1961). Cleaning out ditches can make other poisonous plants more accessible to stock; one such would be hemlock water dropwort (*Oenanthe crocata* L) (Forsyth 1968) whose toxic roots have been mistaken for parsnips. Such events are far from rare and a 1978 study of the 17 Western States in the USA estimated that the economic

impact of poisonous plants amounted to $107 million per year (Nielson 1978).

On farm, the plant's natural defences against disease may be supplemented by pesticide treatment. In the developed world this practice is closely controlled by legislation so that only approved chemicals are applied at recommended rates by accredited practitioners, and maximum residue limits are set to reflect good practice rather than the border between safe and unsafe levels. In EU legislation the limits for pesticide residues in feed are listed alongside those for all undesirable substances (EC, 2002a). However, such controls do not satisfy everyone; the declared aim of organisations such as the Pesticide Action Network is to reduce the use of what they term 'hazardous pesticides' (PAN 2003), while the organic movement is opposed to all pesticide use.

Pasture plants may be affected by fungal disease with potentially devastating effects on the grazing animal. Ergot is possibly the most widely known fungal problem and is caused by an infection of cereals and grasses by spores of the Claviceps fungus. This fungus proliferates within the target plant before millions of spores are dispersed into the immediate environment where they infect neighbouring plants. At a later stage the ergot resting body, the sclerotium, becomes visible on mature plants and this body contains a number of toxic alkaloids. When an animal eats the contaminated crop these toxins are consumed and they may lead to a number of serious problems, particularly to the animal's blood circulation and nerve transmission (Eadie 2003).

Facial eczema has been a serious problem in New Zealand sheep and cattle and is caused by spores of the fungus *Pithomyces chartarum*, which lives in the base of the sward (Dinger 1999). When eaten by stock these spores release a toxin that attacks the liver and depresses both the fecundity of the dams and the growth rate of their offspring.

Mould species are important agents in the natural process of decay, but some also invade feed and food crops. Under certain conditions these moulds may produce toxic metabolites (mycotoxins) or antibiotics. Mycotoxins provide a defence mechanism for the mould but they may have a number of serious effects when consumed, ultimately causing cancer, foetal abnormalities, liver and kidney dysfunction, immune suppression and reproductive disorders (as reviewed in Chapter 11). The development of such effects depends on the nature and concentration of the toxin, the duration of exposure and the sensitivity of the animal, and the greatest economic impact of mycotoxins on livestock farms may be due to relatively low inputs that cause reduced animal performance and increased disease incidence. In addition to the mycotoxins that contaminate the crop in the field, some also occur in conserved crops, and in other feeds, when moist aerobic conditions prevail.

Bacterial disease may also occur in animals grazing pasture and botulism, elaborated by *Clostridium botulinum*, has been reported from

countries all around the world. The botulinum toxin is considered by Timbrell (2005) to be second only to ricin in the potency of its effects, though Lax (2005) refers to it as 'the most deadly of all toxins'. Most reported cases have had their origins in the ingestion of carcase material or fodder contaminated with carcase particles, but investigations in The Netherlands (Haagsma and Ter Laak 1978, Notermans *et al*. 1979) have established an infective route via manure and manure-contaminated pasture. The organism has also been found in newly made grass silage, though toxin production is markedly lower at the pH levels typically found in well-fermented silage. By way of contrast, *Listeria monocytogenes*, which is widely distributed in soil and on herbage, can survive in silages of low pH and multiply at the higher pH levels found in poorly fermented silage. Listeriosis in ruminants is a condition that may result in abortion, encephalitis or mastitis and it has been regularly associated with the consumption of silage (Hinton and Bale 1990).

2.4.3 Contamination during storage, transport and processing

Farm crops and other feeds may be contaminated during storage or in transport to the processing or feeding site. This can occur where silos or other storage areas have been inadequately cleaned and the new material comes into contact with the old. The latter may be a moulding residue or a wholly different material. Prominent examples of such incidents have occurred on board ship, as in 1989 when a consignment of rice bran came into contact with a source of lead, and ensuing feed analyses found samples with a lead content as high as 11,550 mg/kg (Adamson 1990). Of topical significance is the possibility that the contaminant may be a GM variant that has yet to receive authorisation for use as a feed in the EU, which would render the whole consignment unacceptable in Europe (reviewed in Chapter 19). Provided that such inadvertent mixing has been avoided, the storage conditions and the moisture content of the material (strictly the moisture activity) need to be appropriate if deterioration in store is to be precluded. Similar concerns also need to be considered in relation to the equipment that is used to load and unload the store.

Pest control in stores is a vital consideration because the stored material may attract birds, insects and mammalian intruders. These vermin bring not only a healthy appetite but an unhealthy propensity to foul more feed than they eat, and they may be a vector for disease. For instance, Newcastle disease in poultry has been spread by feral pigeons carrying a variant of the ND virus, which contaminated stored feed with their excreta (Wilson 1986).

Other major virus diseases, such as foot and mouth, swine fever and swine vesicular disease, have been transmitted by feed when contaminated animal products have been incorporated in the diet (Hinton and Bale 1990), but the regulation of rendering practices and the banning of waste foods effectively eradicated such diseases in Europe. However, this largely

satisfactory position was dramatically changed in 1985 when the first case of BSE was reported in UK cattle. It was a disease caused by an infective agent that was most probably spread by its contamination of meat and bone meal, and it eventually resulted in more than 180,000 cases (EAAP 2003).

With the notable exception of salmonella, bacterial infection of feed is not regarded as a major source of pathogens for animals (Hinton and Bale 1990), although *Clostridium botulinum*, as noted above, is the source of pre-formed toxin that may be found in feed. Salmonella is an undoubted source of infection in both animals and man, although Hinton and Bale (1990) reported that feed is not considered to be a principal source of the two serovars most commonly found in UK disease incidents (*S. Dublin* and *S. typhimurium*).

Contamination can occur during processing and dioxin contamination is of particular note because chemicals in this group are highly toxic (Timbrell 2005 and reviewed in Chapter 8). They are also widespread in the environment as a consequence of their production during the manufacture of herbicides, wood preservatives and paper and the burning of wastes, particularly the incineration of plastics. Dioxin contamination of feed has occurred when contaminated materials were used during processing. This was the case in Brazil during 1997/98, when contaminated lime was used in the production of citrus pulp pellets (ESF 2000) and 100,000 tonnes of this feed, destined for 11 European countries, had to be destroyed. A further incident took place in the Netherlands when contaminated marl clay from a German quarry was used in a potato processing operation (Veerman 2004). Investigations demonstrated that the contamination was confined to the peel-containing fractions that were used for animal feed and was not found in the food products produced from peeled potatoes. However, 140 farms were affected and dioxin did reach the food supply via milk from cows fed the contaminated feed. Dioxin contamination has also taken place when feed has been dried in direct flame equipment and the product came into contact with contaminated fumes. This occurred in Ireland in 2008 when processed bread was being dried. The contaminated feed was then fed to pigs with the consequence that all Irish pork was recalled from sale (Reilly 2009).

2.4.4 Contamination by malpractice

In addition to the many inadvertent ways in which the feed and food chain may be contaminated, it is possible for contaminants to be introduced deliberately.

Toxic wastes may seem expensive to dispose of by an approved route, and it may seem cheaper and simpler for operators willing to operate outside the law – and with no regard to the potential consequences – to add the waste to material that is destined for another purpose. This is how transformer oil contaminated with dioxins and furans came to be added to

a batch of animal fat in Belgium in 1998. The initial amount of contaminated product has been variously stated as 4 kg (Ziggers 1999) and 25 litres (ESF 2000), but those amounts are relatively small in comparison with an estimated 14,000 tonnes of feed into which the contaminated fat was then incorporated. This feed was then supplied to more than 500 poultry farms, 1600 pig farms and 400 cattle farms in Belgium, the Netherlands and France. Widespread disease and death occurred amongst the chickens, and a range of contaminated meats, eggs, dairy products and baked goods entered the food chain.

In a parallel incident in 2002 a contraceptive product, medroxyprogesterone acetate (MPA), was disposed of in Ireland by adding it to a quantity of glucose syrup that was then incorporated in pig feed. The contamination was not discovered until a fertility problem was investigated in pigs in The Netherlands (FSA 2002, 2009).

2.5 Rapid alert to feed and food contamination threats

A European rapid alert system for food contamination incidents was put in place more than 30 years ago, but it was not until 1999 – as a consequence of the disastrous dioxin problem in Belgium and neighbouring states – that the need to cover the whole food chain was recognised (RASFF 2009). The European alert system became involved in this problem only when dioxins were discovered in meat and not when the animals first became sick. This delay exacerbated an already serious situation and, ultimately, six million chickens were culled and a wide range of contaminated foods had to be recalled from retail outlets for incineration at high temperature.

In 2002, the international network that now links the food and feed control authorities in 31 countries across Europe was consolidated in EU legislation (EC, 2002b, RASFF 2002). In addition to EU Member States the network extends to Iceland, Liechtenstein, Norway and partially to Switzerland. Whenever a risk to human health is identified in either food or feed – either within the region or at a port of entry – rapid notification of all member countries is made via the EC Commission. Notification is also made to any third parties involved and, helpfully, relevant technical information is supplied that will enable the recipients to take appropriate action to manage the risk.

FEFAC, the European Association of Compound Feed Producers, has extracted the animal feed data from RASFF Annual Reports and, although their figures show feed to be a relatively minor contributor to the total of feed and food incidents, there was an increasing trend up until 2008 – see Table 2.4. The most frequently reported problem was microbiological contamination, with the salmonella group being by far the most common pathogen found. The range of contaminants found in livestock feeds (i.e. excluding pet food) is shown in Table 2.5.

Table 2.4 Feed[1] notifications to RASFF[2]

	2004	2005	2006	2007	2008	2009
No. of feed notifications	65	85	129	167	212	205
Feed as % of total notifications	2.5	3.0	4.4	5.6	6.8	6.4

[1] All types of feed notification including pet food. Feed information extracted from RASFF data by FEFAC (2010).
[2] European Rapid Alert System for Feed and Food.

Table 2.5 Contaminants found in livestock feeds[1]

	2007	2008	2009
Microorganisms	59	81	81
Prohibited substances	10	38	5
Dioxins and PCBs[2]	11	11	5
Mycotoxins	12	12	8
PAPs[3] and bones	14	8	18
Heavy metals	5	4	9
Unauthorised GM events	6	1	16
Others	16	5	5

[1] Extracted from RASFF data by FEFAC and excludes pet foods.
[2] PCBs are polychlorinated biphenyls.
[3] PAPs are processed animal proteins from animals fit for human consumption.

Given the global dimension of food safety it is notable that there are proposals to extend the European rapid alert system into other parts of the world (RASFF 2009, De Felipe 2009). Discussions have been held with the World Health Organisation with the aim of mounting and coordinating similar systems in other regions and forming links with other systems like the WHO's International Food Safety Authorities Network (INFOSAN).

Contamination may not always be avoidable, but a rapid alert system facilitates a rapid response and improves the likelihood of reducing the impact on feed and food safety.

2.6 Future trends

There is a growing demand for meat, milk and eggs in developing countries and, coupled with the expected increase in population, this is forecast to lead to a near-doubling of the world meat demand by 2050 (FAO 2006). That will be difficult to sustain given the existing demand for grazing land and feed-crop production, and the inescapable consequence will be intensifying pressure on the animal feed supply. During this period one may also

expect to see increasing signs of global warming, with floods and prolonged droughts affecting previously productive farmland. The impact of diverting food crops towards biofuels and burning co-product feeds as renewable energy sources will also increase the pressure. A recent United Nations report (UNEP 2009) confidently suggests that the world would be able to sustain the projected demands of population growth, and a 50% increase in aquaculture, by increasing food energy efficiency ... in their words 'by developing alternatives to cereals in animal feed such as by recycling waste and using fish discards'. This is a challenging target and it is clear that much care will be needed to ensure that such developments do not compromise feed and food safety.

2.7 Sources of further information and advice

Feed legislation may make heavy reading but the preamble sections provide confirmation that feed safety is deservedly a high priority, and that new pieces of legislation are introduced to remove loopholes that may potentially threaten safety. The websites of the European Food Safety Authority (EFSA) and national authorities (such as the Food Standards Agency in England) and of the European Rapid Alert System provide regular updates of contamination incidents, and their Annual Reports provide useful summaries. In the aftermath of substantial incidents it is always useful to draw up a comprehensive report of the causes, the impact and the changes that must be implemented. No contamination incident has been bigger than BSE and that crisis spawned many reports, but the forward-looking report produced by the European Association for Animal Production (EAAP 2003) on the impact on the European livestock sector was a model of its kind.

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3

The ecology and control of bacterial pathogens in animal feed

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Abstract: Animal feed is vulnerable to the introduction of bacteria along the entire production chain. The presence of pathogens in feed can arise due to usage of contaminated raw ingredients, or due to contamination during transportation, at the feed mill, or on-farm. Since bacterial contaminants are not uniformly distributed in feed, bacteria that are present may be damaged or injured and difficulties may arise during microbial analysis; laboratory testing of feed and feed ingredients may produce false negative results. The goal of pathogen control in feed should be to make sure that feed contaminants are below a critical threshold to minimize the risk to human and animal health. Reducing the incidence of pathogens in feed requires both improvement of programs to monitor feed production and the use of sensitive and rapid pathogen detection methods. It also requires intervention strategies including chemical and physical treatments to reduce and/or eliminate multiple and/or specific feedborne pathogens.

Key words: feedborne, *Salmonella*, feed, sampling.

3.1 Introduction

Animal feed is considered one of the main carriers of bacterial pathogens in food animal production. Animals that consume contaminated feed could get infected and colonized with pathogens, resulting in pathogen shedding in the farm environment comprising a risk for the entire animal unit of flock. Animal feed is vulnerable to the introduction of bacteria at the pre-harvest stage (i.e. raw feed ingredients such as plant materials and processed animal proteins may become contaminated), at harvest (during production at the feed mill), and postharvest (during transportation and dispensing at the farm). In 1990, the United States Food and Drug Administration (FDA) set a goal to make animal feed and feed ingredients *Salmonella*-free (Mitchell and McChesney, 1991). Although both the

livestock and poultry industries realize the role and importance of feed in spreading foodborne pathogens in agriculture, contamination is still a widespread problem in the industry and control and/or elimination of contaminants continues to be a daunting task.

Ensuring that feed samples are pathogen-free remains a major challenge, as (1) contaminants are not uniformly distributed in feed, (2) organisms might be damaged or injured, making them difficult to isolate/detect, (3) microbiological and molecular laboratory methods have detection limits, and (4) deciding on the number of samples to collect for microbial analysis and the sample volume is not straightforward. That being said, the goal in pathogen control in feed should be making sure that feed contaminants are below a certain level (i.e. below a certain threshold) which will pose minimal risk to animal and human health. This can be achieved by implementing 'sound and practical' microbial sampling plans and Hazard Analysis Critical Control Point (HACCP) programs, in addition to applying intervention strategies such as chemical and physical treatments to control pathogens in feed.

3.2 Foodborne pathogen contamination in animal feeds and ingredients

Animal feed has been found to be contaminated with a variety of pathogens such as *Salmonella* spp., *Escherichia coli* O157:H7, and *Enterococcus* (Cox *et al*., 1983; Davies and Wray, 1997; Davis *et al*., 2003). Animal feed manufacturing and distribution is a multi-stage process and bacterial contamination can occur at various stages. In the case of plant-based feeds, these include the following: crop growth in the field (i.e. the preharvest stage), harvesting, processing at the feed mill (e.g. grinding, mixing pelleting), storage at the mill, transportation to animal farms, storage at the farms and dispensing to animals for consumption. Control of pathogens in raw feed ingredients and during feed production has a significant impact on feed contamination.

3.2.1 Preharvest feed contamination

Raw ingredients such as plant materials (e.g. grains, corn and soybean) may become inoculated with bacterial contaminants carried on soil particles that travel with the wind, as well as those found in contaminated irrigation water and animal feces used as fertilizer for the crops (Maciorowski *et al*., 2004). Soil is a complex ecosystem that contains a wide range of diverse microbial populations. Soil can be a recipient of enteric pathogens such as *Salmonella* spp., *E. coli* O157:H7 and *Campylobacter* from the fecal materials of both animals and humans and from the solid wastes of food processing plants (Santamaria and Toranzos, 2003). Therefore,

contaminated soil is a risk factor in the ecology of feed contamination. Furthermore, irrigation water contaminated with pathogens is an indirect source of feed contamination through plant materials used in feed manufacture. Several studies have reported the presence of enteric pathogens in irrigation water used for raw fruits and vegetables (Islam *et al*., 2004a; Beuchat, 1999, 2002). However, very limited information is available on the prevalence and levels of enteric pathogens on the surfaces of soybean and corn plants pre- and postharvest. One report by Lewis *et al*. (2007) found that imported young corn was contaminated with *Shigella*. The use of manure as a fertilizer is a common practice in the US and around the world. Land application of animal manure and poultry litter can lead to the introduction of enteric pathogens into livestock and poultry feed through contaminated plants. Proper manure composting prior to land application may kill pathogenic bacteria such as *Salmonella* and *E. coli* O157:H7 (Edrington *et al*., 2009; Islam *et al*., 2004b). However, enteric pathogens can survive in feces and manure-amended soil for long periods of time and the soil itself can, in turn, become a source of inocula. Therefore, controlling pathogen populations in animal manure used for land application is important in reducing the risk of pathogen contamination in plants used for feed production.

3.2.2 Postharvest feed contamination

At the *feed mill*, animal feed can also become contaminated during feed manufacture or processing as a result of cross-contamination. The most critical point for microbial contamination at the mill is the post-processing heat treatment process. The heating process is required to pellet the feed and usually kills most of the pathogens in the produced feed, but inadequate operating temperatures for the pelleting equipment and feed conditioner are risk factors. Contamination of feed before and after the heating point is common and can be attributed to many factors within feed mill facilities. These include unclean receiving and unloading areas, unclean intake pits, dust generated by the feed ingredients, dirty conveyers with leftover feed from previous loads, inadequate feed storage conditions, and presence of non-employees or visitors in unsanitary clothes (Jones and Ricke, 1994; Jones and Richardson, 2004). Feed ingredients should be inspected prior to unloading for signs of rodent contamination, bird droppings or insect infestation. Inspection is particularly important given the difficulty of testing large amounts of incoming ingredients or of testing the feed during and after production due to the associated cost, time and labor. Therefore, feed mills should consider investigating which suppliers have consistently and reliably delivered ingredients without macro- or micro-levels of contamination. Furthermore, sampling plans designed to determine the minimum number of sampling units required to represent the microbiological quality of feed should be considered when needed.

In early studies, Hacking *et al.* (1978) detected *Salmonella* in 3% ($n = 111$) of pelleted feed sampled from one commercial poultry feed mill in Canada, whereas Cox *et al.* (1983) did not find *Salmonella* in pelleted poultry feed from 10 commercial feed mills in three southeastern US states. The latter authors detected *Salmonella* in mash feed (58%; $n = 26$) as well as in meat and bone meal samples (92%; $n = 13$). These data clearly indicate the level of reduction that can be achieved by feed processing, including the heating step. In the United Kingdom, nine feed mills that produced a variety of animal and poultry feeds were sampled over time for the presence of *Salmonella* (Davies and Wray, 1997). The authors found *Salmonella* not only in the finished products but also in feed mill equipment, including intake pits (24.1%) and the cooling systems (20.2%). Furthermore, *Salmonella* was isolated from fresh wild bird droppings collected from the intake pit areas, warehouses, and unloading areas. Jones and Richardson (2004) visited three feed mills and collected samples from raw ingredients, the mixer and pellet mill, pellet coolers and the finished product. The authors concluded that feed raw ingredients and dust were the main sources of feed mill contamination with *Salmonella*. They reported a *Salmonella* prevalence of 8.8% ($n = 178$) and 4.2% ($n = 451$) in mash and pelleted feed samples, respectively. A higher prevalence of *Salmonella* was observed in dust samples compared to the actual feed samples within each sampling area (Jones and Richardson, 2004).

In general, most of the studies reported earlier in this section have lacked a strong temporal component to assess both patterns of pathogen contamination over time (i.e., seasonality) and changes in microbial populations over time. Furthermore, epidemiological studies are needed to determine the relationship between potential risk factors at the feed mills that contribute to the prevalence and level of pathogen contaminants in the feed production process.

During *transportation to the farms*, feed is susceptible to the introduction of pathogens and subsequent survival and growth of the organisms. Unclean transportation containers, traces of previous contaminated feed in transportation trucks, and changes in temperature and humidity during transportation and/or storage are all risk factors for the introduction and survival or growth of pathogens in animal feed. Fedorka-Cray *et al.* (1997) isolated *Salmonella* from trucks transporting swine feed (0.7%, $n = 549$) and from feed samples taken from those trucks (23.5%, $n = 17$). The European Union (EU) Department for Environment Food and Rural Affairs (DEFRA), Code of Practice for the Control of *Salmonella* (2009) recommended that feed should be transported in vehicles and containers used to carry dry products to avoid any pre-existing moisture. Moreover, it was recommended that vehicles used to transport feed should be subjected to cleaning and sanitation to ensure no waste build-up and cross-contamination from previous feed loads occur.

At the farm, feed is usually stored in large bins outside livestock pens, feedlots, and poultry houses. Feed storage at the farm in unclean

environments and in defective storage bins may introduce pathogens and/or propagate resident pathogens in the stored feed. Feed should be stored in closed bins that do not share common airspace with the livestock or poultry operations. All storage areas should be emptied and cleaned regularly according to type and condition of feed stored. It is important to keep the feed dry to prevent growth of contaminants such as *Salmonella* and *E. coli* O157:H7 that require moisture to multiply. Pathogens survive differently under various temperatures and water activities. In order to survive in feed, *Salmonella*, for instance, must combat the same environmental conditions as the nonpathogenic microflora. In 1978, Williams and Benson observed that low water activity (0.43) was not completely effective in destroying *Salmonella* populations in feed. In another study, Juven *et al*. (1984) demonstrated that the survival of *Salmonella* was greater at a water activity of 0.43 than at one of 0.75. Several investigators observed the survival and heat resistance of *Salmonella* in meat and bone meal, and in poultry feed, to be inversely proportional to moisture content and relative humidity but not to the type of protein in the feed or to organic versus conventional poultry feed (Liu *et al*., 1969; Carlson and Snoeyenbos, 1970; Juven *et al*., 1984; Ha *et al*., 1998; Petkar *et al*., 2011). Plant-based protein meals also do not appear to reduce colonization and shedding of *Salmonella* Heidelberg in broiler birds (Alali *et al*., 2011).

3.2.3 Environmental sources of pathogens

Bhatia *et al*. (1979) surveyed the environment of broiler houses and concluded that broiler litter was a major vehicle for flock infection with *Salmonella* and that pathogen isolation from litter was a reliable indication of both flock infection and carcass contamination. Infected animals that shed pathogens back to the environment are a cause of horizontal spread of organisms through feed to other animals. Bhatia and McNabb (1980) studied *Salmonella* contamination from the hatchery to the processing plant and found the same *Salmonella* serotype in fluff and meconium samples collected at the hatchery, in litter samples at the boiler farm, and on carcasses following processing. Soerjadi-Liem and Cumming (1984) sampled ceca of broiler flocks for *Salmonella* contamination at the processing plant on a monthly basis for a year and observed more contamination in the colder months than in the warmer months. These researchers also found that birds raised on old litter were contaminated at much lower rates than birds raised on new litter. Lahellec *et al*. (1986) surveyed the French broiler production system and observed that *Salmonella* serotypes originating in the hatchery were less prevalent in the final product than those isolated from the poultry house during the rearing of birds. Goren *et al*. (1988) surveyed the broiler production system in the Netherlands and found a positive correlation between the *Salmonella* isolation rate from litter at 5 weeks of age and the isolation rate from ceca at processing.

Dougherty (1976) followed *Salmonella* contamination in broiler flocks and reported that up to 37.5% of the day-old chicks in his study were contaminated with *Salmonella*, but the contamination rate decreased to approximately 5.0% on the farm just prior to processing. Dougherty (1976) also found that the *Salmonella* serotypes present in feed ingredients were not the same as those in broiler flocks and suggested that contamination in broiler flocks may have originated either from breeder-multiplier flocks or from the hatchery. Nevertheless, in a survey of the Australian broiler production system, MacKenzie and Bains (1976) found that 82% of the *Salmonella* isolated from broiler carcasses could be traced to feed or feed ingredients. However, they did not examine day-old chicks or breeder hens for *Salmonella*.

Jones *et al*. (1991) surveyed *Salmonella* contamination in the broiler production and processing systems in the US. The authors observed similar rates of contamination in cecal droppings collected from breeder houses, day-old chicks and live birds in broiler houses and suggested that the breeder house environment is an important vehicle for the transmission of contamination from feed to the final product. Jones *et al*. (1991) also underlined the need for a comprehensive approach to *Salmonella* contamination control in broiler production and processing systems. This includes specific HACCP approaches for the control of *Salmonella* in feeds as outlined by Jones and Ricke (1994).

Feedlot cattle feed has been reported to harbor *E. coli* O157:H7 (Dodd *et al*., 2003). The authors collected samples (n = 504) from feed bunks from 54 feedlots in four US states. The prevalence of *E. coli* O157:H7 was 14.9%. Interestingly, the authors found that there was no significant relationship between coliform counts in feed and *E. coli* O157:H7 prevalence. The authors recognize that potential risk factors for feed contamination with *E. coli* O157:H7 could be feed components, calves shedding the pathogen when entering the feedlot, and manure handling practices.

3.2.4 Pathogens in specific protein supplements

Animal and plant protein meals used in feed production can serve as vehicles for the introduction of contaminants to the corresponding feedstuffs (McChesney and Kaplan, 1998; Wagner, 2004; Myint *et al*., 2007). Wagner (2004) reported *Salmonella*, *Enterococcus*, and *E. coli* contamination in animal protein (n = 122) and plant protein samples (n = 79). In the animal protein samples, the prevalence of contamination was 34%, 84% and 40%, respectively; whereas in plant protein samples, the prevalence was 5%, 91%, and 43%, respectively.

Pathogen contamination of vegetable proteins from cereal grains has been well documented (Crump *et al*., 2002; Jones and Richardson, 2004; Davies *et al*., 2004). Soybean meal and corn meal are the most common vegetable protein supplements in the US livestock and poultry industry

(Cullison and Lowrey, 1987; Jurgens, 1993). Other protein meals such as canola are used, but on a much more limited scale. Jones and Richardson (2004) found that 10% ($n = 10$) of soybean meal, 100% ($n = 2$) of cottonseed meal, and 5.26% ($n = 19$) of corn samples contained *Salmonella*. In contrast, Myint *et al*. (2007) reported a very low *Salmonella* prevalence: one isolate in sunflower plant protein meal. The authors tested 158 samples of a variety of plant proteins including soybean meal, maize, cottonseed meal, sunflower, and peanuts that were purchased from different commercial feed stores in the US. The overall *Enterococcus* and *E. coli* prevalences were 80% and 9%, respectively. The highest prevalence for both bacteria was in maize samples (Myint *et al*., 2007). MacKenzie and Bains (1976) reported presence of *Salmonella* in vegetable proteins including sunflower meal, peanut meal, bran meal, soybean meal, and corn meal. Sunflower meal samples were highly contaminated with *S*. Birnum, whereas soybean meal samples were highly contaminated with *S*. Singapore. Hacking *et al*. (1978) found *Salmonella* in corn and soybean meal samples collected from one feed mill in Ontario, Canada. The authors did not serotype the isolates.

In Japan, Morita *et al*. (2003, 2004) reported that soybean and canola seeds were contaminated with *Salmonella* when brought to the oil manufacturing plant. Oil is extracted from the seeds and the remainder is the protein meal used in feed production. The authors concluded that *Salmonella* might contaminate the plant and consequently the protein meals. They suggested that in order to prevent the spread and survival of *Salmonella*, it is important to reduce oil meal plant contamination. Morita *et al*. (2006) demonstrated that oil meal plant workers, dust and rodents were the most significant risk factors that contributed to the contamination of oil manufacturing plants where protein meals are produced.

3.2.5 Contamination of animal byproducts

Animal byproducts are a large source of protein in the diets of animals fed on manufactured feed products. Fish meal, blood meal, feather meal, and meat and bone meal are common types of animal byproduct protein meal used in the US and worldwide. Contamination can occur during production of these meals, and in turn pathogens can be carried forward to the feed (Gabis, 1991; Nesse *et al*., 2003). Hacking *et al*. (1978) examined samples of meat meal and feather meal for *Salmonella* and found them to be contaminated at prevalences of 81% ($n = 21$) and 40% ($n = 15$), respectively. Based on their study, the authors concluded that meat meal was the primary source of feed contamination. In another study, Hofacre *et al*. (2001) isolated *Salmonella* and various coliform bacteria at prevalences of 14% and 23%, respectively, from meat and bone meal samples ($n = 43$) at two poultry companies' feed mills. The authors observed that 'blended' protein meal (rendered fish, cattle and/or poultry) samples had lower *Salmonella* and coliform percentages – 5% and 16%, respectively – compared to meat and

bone meals (Hofacre *et al*., 2001). Isa *et al*. (1963) collected feed ingredient samples and tested them for the presence of *Salmonella*. The authors recovered *Salmonella* spp. in 31% of meat meal samples (n = 84). Several *Salmonella* spp. were identified including *S.* Bredeney, *S.* Seftenberg, *S.* Montevideo, *S.* Kentucky, and *S.* Heidelberg. *Salmonella* spp. were also recovered from fish meal, bone meal, and blood meal at 9.1% (n = 11), 60% (n = 10), and 15.4% (n = 13), respectively. The percentages reported here should be interpreted with caution since the number of samples was small (n < 11) compared to the meat meal (n = 84) (Isa *et al*., 1963). A similar high *Salmonella* contamination percentage in meat and bone meal (31%; n = 83) was reported by Veldman *et al*. (1995) in the Netherlands. However, since 2000, the European Union has banned the import and export of meat and bone meals. Although the level of pathogen contamination in feed that represents a consistent public health risk is undefined, studies have attempted to link contaminated feed to human infections. In one study by Clark *et al*. (1973), 507 cases of *S.* Agona infections in the US were linked to contaminated Peruvian fish meal that was used in poultry feed in Mississippi chicken farms.

3.3 Detection of pathogens in feeds

Presently, a farm or feed mill in the US may adopt several good manufacturing practices (GMPs) to reduce feed recontamination. Feed bins, feed pans, cross augers, hoppers, silos and transport trucks may be regularly cleaned and painted with ceramic paint to prevent the buildup of caked feed that may be contaminated with pathogenic molds, bacteria, or mycotoxins (Hamilton, 1975; Hess, 1994). Systems are in development which may disinfect truck tires while the truck is still moving, reducing soil contamination between the farm and feed mill (Best, 1994). Equipment may be manually scrubbed between shifts, as scrubbing has been shown to reduce the attachment of *E. coli* O157:H7 to metal surfaces (Farrell *et al*., 1998). Monitoring *Salmonella* spp. in either feed mixtures or feed ingredients will probably require some sort of direct detection of *Salmonella* spp. Dust in feed mills may also be sampled for airborne *Salmonella* spp., giving a general indication of *Salmonella* spp. presence in the environment that may reduce the problem of sample size (Kwon *et al*., 2000) discussed further in Section 3.5 below.

3.3.1 Conventional and rapid methods

Numerous detection methodologies have been examined over the years for quantifying salmonellae in feeds and many have proven to be effective for *Salmonella* isolation and detection in a variety of feeds (Ha *et al*., 1995a, b; Williams, 1981; Ricke *et al*., 1998; Maciorowski *et al*., 2004; 2006a, b). A

number of traditional culture methods have been employed, which rely on the use of pre-enrichment media such as lactose broth or universal enrichment broth, followed by selective media for bacterial enumeration, or enrichment broth combined with selective media when bacterial numbers are expected to be low. Characterized colonies are usually stab-inoculated into differential media and then biochemically characterized using chemical tests (e.g., indole test). Isolates are confirmed by serological typing with somatic (O) and flagellar (H) antisera. The culture-based methods have been discussed in detail elsewhere (Williams, 1981; Maciorowski *et al*., 2004, 2005, 2006a). Given the potential need for detection sensitivity, molecular detection technologies may be the best candidate for developing rapid sensitive methods for identifying small numbers of *Salmonella* in the background of large volumes of feed.

Several studies have been done using polymerase chain reaction (PCR) assays and commercial kits to detect *Salmonella* spp. in a wide variety of feed sources (Ricke *et al*., 1998; Maciorowski *et al*., 2000, 2005; Löfström *et al*., 2004). Furthermore, real-time PCR assay has been used to detect *Salmonella* in animal feed (Bohaychuk *et al*., 2007; Nam *et al*., 2005). In addition, DNA array technology has recently been utilized to track the dissemination of a specific *Salmonella* serotype in Spanish feed mills (Alvarez *et al*., 2003). A simple and rapid method has been developed using P22::luxAB bacteriophage to detect *Salmonella* spp. in poultry feed (Thouand *et al*., 2008). The primary difficulty with routine application of molecular assays is the problem of extracting and recovering representative samples from feeds for molecular analyses (Maciorowski *et al*., 2005). As more commercial assays become available standard protocols for extraction, enrichment and PCR can be more systematically evaluated to determine the efficacy, the detection limits, and the minimum enrichment time required for routine analyses of animal feeds. It needs to be noted, however, that the interpretation of the PCR results needs to be realistic, as refined methods of extraction for PCR analysis will also detect nonviable bacteria, which are of no risk. This is one of the major pitfalls in the control of decontaminated feed material.

3.4 Persistence of *Salmonella* in feed

Besides the quantitative enumeration, the physiological status of the pathogens, including salmonellae, is of importance. Many environmental stress factors modulate the virulence of potential pathogenic bacteria. *Salmonella* and other foodborne pathogens become virulent when exposed to different stress factors such as non-optimal pH, oxygen availability, and osmolarity (Mekalanos, 1992; Foster and Spector, 1995; Bajaj *et al*., 1996; Nutt *et al*., 2003a). In addition to the synthesis of toxins and survival factors, virulence is expressed via production of a variety of attachment and invasion proteins that interact with the intestinal cells (Darwin and Miller, 1999).

It is conceivable that *Salmonella* strains exposed to the relatively harsh environments associated with dry poultry feeds will change (and increase) their virulence. In turn, treatment methods such as acid, heating and irradiation have the potential to reduce the number of *Salmonella* in feedstuffs, but such treatments may also increase the levels of virulence in the remaining viable cells. Although this has not been examined directly in poultry feeds, some evidence to support this hypothesis does exist in that in *S.* Typhimurium an increase in the expression of virulence genes was observed after long-term exposure to poultry drinking water and to extracts from some food matrices (Nutt *et al*., 2002, 2003b). Feed processing may also directly influence the virulence status of *Salmonella* contaminants. Therefore it is recommended to assess *Salmonella* virulence gene expression during and after feed processing (Maciorowski *et al*., 2005). A candidate gene for quantifying virulence expression in *Salmonella* is the gene *hilA* (hyperinvasive loci) which produces a transcriptional activator that coordinately regulates the expression of invasion genes in *Salmonella* in response to environmental conditions including stress (Bajaj *et al*., 1996). A series of *in vitro* and *in vivo* experiments consistently indicate a positive relationship between increased *hilA* expression and increased *Salmonella* infection in susceptible chickens (Durant *et al*., 1999, 2000; Dunkley *et al*., 2007). Therefore *hilA* response could be an important indicator of the potential for poultry feed environments and processing conditions to increase the pathogenicity of contaminating *Salmonella*.

If it were possible to quantify the virulence in *Salmonella*, a more accurate assessment of the potential infectivity of the pathogen for different feeds and feed processing conditions would be possible. It is conceivable that approaches such as real time (RT)-PCR assay could be used to quantify *Salmonella* virulence status; a positive result could enable the prediction of the bird infection risk when associated with a certain level of poultry feed contamination. The results of such a test could also provide information that could be used to improve HACCP programs by identifying feed processing steps and storage practices that might lead to an increased risk of birds becoming infected. Furthermore, this information could be used by the industry to design preventative strategies at the hatchery and breeder flock stage which included feeds among the control measures.

3.5 Sampling plans to control pathogens in feed

One of the goals of the US FDA is to ensure that animal feed is safe and poses no or minimal risk to human health when fed to food-producing animals. In 1990, the FDA Center for Veterinary Medicine (CVM) set a goal that animal feed and animal feed ingredients must be *Salmonella*-free. However, at the current time the feed industry continues to struggle with the control and elimination of *Salmonella*, especially during production at

the feed mills (Davies and Wray, 1996, 1997; Whyte *et al*., 2002), and the FDA-CVM goal seems far from being reached, especially under current animal feed production systems. Improved strategies for control and prevention of feedborne pathogens are needed. Both the FDA and the USDA have no regulations on feed testing for human foodborne pathogens. Therefore, the majority of feed mills have a limited HACCP program or none at all and no sampling plans in place for the detection of pathogens in either finished feed or raw feed ingredients. In contrast, in some parts of the European Union (EU) (e.g., Denmark), the *Salmonella* control program in poultry requires that animal feed is *Salmonella*-free. Therefore the Danish feed industry is required to monitor their finish feed and their raw feed ingredients, and to use 'good production practices' to eliminate *Salmonella* presence in the feed. Many poultry companies in the US with breeder flocks have themselves established a 'zero tolerance' policy for presence of *Salmonella* in the produced feed as part of their *Salmonella* control program, often screening feeds themselves for pathogens (mainly *Salmonella*) before they are shipped to breeder farms. These companies usually collect multiple samples (up to 10) from finished feed trucks during loading at the feed mill or unloading at the farms. Samples collected are usually mixed in a large bag, and then a composite sample of 250 g is obtained and sent to the laboratory for microbial analysis. A similar sampling protocol is also carried out for the raw ingredients during truck unloading at the feed mills.

Despite the variability in levels of application worldwide, the existence of a well-designed HACCP system at a feed mill and the monitoring, testing, and analysis of critical control points during feed production are very important in minimizing the contamination and recontamination of feed. One of the major limitations of the HACCP system in the feed mill, though, is the need to implement sampling plans for screening large amounts of raw ingredients and finish feed for presence of pathogens (Jones and Ricke, 1994). A feed mill produces on average 40 tons (80,000 lb or 36,320,000 g) of feed per hour. Jones and Ricke (1994) calculated that feed produced in an hour has 36.32 trillion potential sites for *Salmonella* contamination, assuming that 1 million organisms can exist in every gram of feed. Given that *Salmonella* is likely present at very low numbers in feed (less than 1 bacterium per gram of feed according to D'Aoust and Sewell (1986) and *Salmonella* presence is not uniformly distributed, then collecting '*n*' number of feed samples for microbial detection of *Salmonella* would be like 'looking for a needle in a very large haystack'. Furthermore, since it usually takes a few days to get the laboratory results of the feed microbial testing (using culture methods), by that time most of the feed produced has been already shipped out.

Given the large amount of feed produced at any feed mill, cost-effective sampling plans with an appropriate degree of confidence need to be put in place. Microbial sampling plans for feed have been somewhat arbitrary in the past. Although many sampling plans that have been designed are not

statistically based, one can test the reliability of these plans in comparison to statistically based plans. Each sampling plan must define the number of analytical test units (i.e., sample size) '*n*' and an acceptance number '*c*' for microbiological quality control. If the sample *n* has more than *c* defective units, the corresponding lot should be rejected. Otherwise (if the number of defective units ≤ *c*), the lot then should be accepted. A 'lot' is defined as a quantity of food or feed units produced and handled under uniform conditions. For example, a truckload of feed, or an hour of feed production, can be a 'lot'. The goal for introducing sampling plans is to determine the minimum number of random samples that must be drawn to represent the microbiological quality of a feed 'lot'. To design a sampling plan for a feed, the question one should ask is: What do we want the sampling plan to do for us? And, what are the best sampling locations? There is a relationship that exists between the number of samples, the variance (homogeneity of the lot), the confidence level (stringency), and the acceptance quality level (percentage of defective sample units tolerated). This relationship can be expressed by the following equation, assuming that a 'lot' of animal feed has an infinite number of pellets or particles where pathogens can reside:

$$n = \frac{\ln(\alpha)}{\ln(1-p)}$$

where

n = required sample size
$\alpha = 1 -$ desired level of confidence
p = expected probability of defective samples in a 'lot' of feed.

There are no sampling plans that can ensure that feed is *Salmonella*-free regardless of the number of samples collected and tested. Therefore, the concept of zero tolerance (c = 0) is neither feasible nor practical for the feed industry. Assuming that the probability of a defective unit in a lot of feed is 1%, which is greater than 'c = 0', then based on the above equation, the number of sample units needed to ensure that less than 1% of the lot is contaminated with *Salmonella* (defective) 90% of the time is 298. Table 3.1 shows the sample size for different levels of confidence and probability of defective units. When designing a sampling plan, one should consider what is economically practical and the amount of time and labor required to collect and analyze the samples. The large number of samples in the above example (n = 298), even when considering compositing the samples into a lower number, still will be a significant burden in terms of cost, labor, and time to the feed industry.

3.5.1 Alternative sampling plan to detect pathogen in feed

There is no universal robust sampling plan that will work for all feed mills. Rather, each feed mill or poultry company must develop their own plan(s)

Table 3.1 Number of sample units required for several confidence levels in detecting at least one defective sample unit (c = 0) in lots with defective probability ranging from 0.1% to 50

Percent defective in a 'lot'	Desired confidence level (%)						
	50	60	70	80	90	95	99
0.1	693	916	1204	1609	2302	2994	4603
0.5	139	183	241	321	460	598	919
1	69	92	120	161	230	298	459
5	14	18	24	32	45	59	90
10	7	9	12	16	22	29	44
50	1	2	2	3	4	5	7

to fit with their manufacturing practices, hazard production points, type of feed produced, and target pathogen(s). For *Salmonella*, a possible practical sample size is 59 (200 g each) individual samples collected from finish feed and the raw ingredients to ensure that a 'lot' is accepted 95% of the time if the contamination level ≥5%. The assumption is that levels of contamination less than 5% might not pose as high a risk of *Salmonella* transmission to animals compared to higher levels of contamination. We recommend that roughly 4 grams from each individual sample can be taken to form a composite sample of ~250 g to be used in laboratory analysis for *Salmonella*. The effectiveness of microbial sampling in feed can be assessed using quantitative risk assessment models as described in Duffy and Schaffner (2002). A simulation model can be constructed using quantitative pathogen data (e.g., CFU/g) from raw ingredients and finished feed to determine if the sampling plan is efficient in reducing the number of pathogens between the incoming and outgoing products. The model described by Duffy and Schaffner (2002) uses a Monte Carlo simulation method in Analytica® software (Lumica Inc., Los Gatos, CA). A limitation to this risk assessment method is that the model does not work with low levels of contamination.

3.6 Reduction and/or elimination of feedborne pathogens

3.6.1 Feed additives to control pathogens in the feed

Reduction or the total elimination of *Salmonella* spp. in animal feed is a current, ongoing subject of research and development, with most efforts focusing on the feed processing stage. Several chemical and physical interventions have been applied to feed to prevent its contamination with *Salmonella* and subsequent colonization of ingested *Salmonella* by animals. These methods include acids such as organic and propionic acids, formaldehyde, heat treatments, and irradiation (Ha *et al*., 1998, 2000; Leeson and Marcotte, 1993; Ricke, 2003; Maciorowski *et al*., 2004). Organic acids have

been added to the feed or to feed ingredients to reduce the levels of *Salmonella*. Combinations of acids and heat treatments are common in the poultry feed industry as they have been proven to effectively reduce the level of *Salmonella* in the end product (Matlho *et al*., 1997).

3.6.2 Feed additives to control pathogens in the animal

Feed additives have been used in animal agriculture to control the colonization of pathogens in animals' guts. As well as being added to feeds and feed ingredients, several organic acids such as formic and propionic acids have been given directly to broiler birds to reduce levels of or eliminate feedborne pathogens from their intestines and prevent shedding of these microorganisms (Thompson and Hinton, 1997; Hinton and Linton, 1988). Prebiotics and probiotics can also be added to feeds as additive substances to reduce or prevent colonization of pathogens in the gut. This topic has been extensively discussed elsewhere (see citations below) and will only be briefly discussed here. Prebiotics are 'nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon' (Gibson and Roberfroid, 1995). Probiotics are single or multiple microorganisms that when ingested by the animal can act as protective microflora in the gut to prevent colonization by pathogens (Ricke and Pillai, 1999; Nisbet, 2002; Ricke *et al*., 2004). Prebiotics and probiotics alter the intestinal microbiota and immune system to reduce colonization by pathogens in the animal gut. Environmental conditions and stress statuses of animals influence the efficacy of prebiotics and probiotics. Lema *et al*. (2001) demonstrated that probiotics reduced fecal shedding of *E. coli* O157:H7 in sheep. In cattle, Brashears *et al*. (2003a, b) demonstrated that a *Lactobacillus acidophilus* culture reduced *E. coli* O157:H7 shedding by more than 50% in finishing cattle. Other studies found that the use of prebiotics can increase the resistance of animals to infection and the incidence of atopic dermatitis (Meyer, 2008).

3.6.3 Feed additives to eliminate colonized pathogen species

One of the main types of feed additive that has been used to eliminate pathogen colonization is antibiotics. Antibiotics have been added to animal feed for the past 50 years in the US and other countries at subtherapeutic levels to prevent disease and promote growth. Antibiotics have been shown to eliminate colonized *S*. Enteritidis in molted laying hens and young chicks (Sato *et al*., 2000; Asakura *et al*., 2001; Holt, 2003). However, the development and spread of antibiotic-resistant bacteria was documented as early as the 1950s (Starr and Reynolds, 1951; Barnes, 1958; Elliott and Barnes, 1959). Furthermore, studies have demonstrated the emergence of multiresistant pathogenic bacteria worldwide in the 1980s (Aarestrup *et al*.,

2003). Consequently, several reports have been published recommending a ban on or reduction of antibiotic use in food animals at subtherapeutic levels as a precautionary measure. Researchers have been interested in finding alternative substances to antibiotics for use in feed. For example, Anderson *et al*. (2005) reported that chlorate and nitro-based feed supplements could reduce pathogen levels in swine, cattle, sheep, and chickens. Natural antimicrobials as present in plant-derived essential oils and their components have showed *in vitro* inhibition of several bacterial pathogens (Kim *et al*., 1995; O'Bryan *et al*., 2008; Callaway *et al*., 2008; Nannapaneni *et al*., 2008, 2009a, b; Chalova *et al*., 2010). Other natural substances such as tannins have also been shown to lower the concentrations of bacteria in the rumen and decrease the amounts of bacterial proteins flowing to the intestine (Waghorn *et al*., 1987). For a detailed review the reader is referred to Chapter 17 of this volume.

3.7 Conclusions

Animal feed is considered one of the primary sources of bacterial pathogens in food-producing animals. Contamination of animal feed leads to infection and colonization of livestock and poultry with pathogens that can be transmitted through the food chain to humans. Animal feed is thus an important early link in the 'farm-to-fork' chain with regard to food safety. Reducing the incidence of pathogens in feed requires improvements in the monitoring of feed production and detection methods for pathogens that are more sensitive and more rapid. It also requires better intervention strategies including chemical and physical treatments to reduce and/or eliminate multiple and/or specific feedborne pathogens. Challenges still exist due to uncertainty about the sources and origins of the pathogens as well as the non-uniform nature of their distribution in feed. Sampling plans are not meant to replace the implementation of HACCP programs at feed mills or from production and harvesting to consumption of feed. Furthermore, sampling plans do not guarantee the absence of pathogens from feed, but should be considered to ensure that contamination of feed does not exceed a level judged to be acceptable.

3.8 References and further reading

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Detection and enumeration of microbiological hazards in animal feed[1]

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Abstract: This chapter provides an overview of methods for pathogen detection and microbiological enumeration that are relevant to implementation of control measures to improve safety to animals and humans that may be applied during the production, storage, distribution and use of animal feed. It does not discuss in detail the procedures that need to be followed to properly apply the methods used to detect and enumerate microbiological hazards in feed and fodder. Instead, readers are encouraged to consult separate texts for further reading on specific methods in common use for detailed descriptions of sampling and analytical procedures.

Key words: microbiological, detection, enumeration, analysis, hazards, pathogens, contamination, animal feed, feedstuffs, fodder.

4.1 Introduction

Although not all human intestinal illnesses caused by microbiological hazards can be traced to animal feed or human food as the origin, the food industry acknowledges that the bacteria most commonly implicated in human intestinal diseases, *Salmonella* spp. and *Campylobacter* spp., may be food-borne in most cases (Andrews *et al*., 2001). Several bacteria such as *Bacillus* spp., *Salmonella*, *Listeria*, pathogenic *Escherichia coli*, and spore-forming clostridia often contaminate grain, feed ingredients and animal feed (Okelo *et al*., 2008). These organisms can be transmitted to livestock through consumption of contaminated feedstuff (Wyatt, 1995) and may ultimately present a risk to animal health, affect the human food supply, or both (Cox *et al*., 1986; Jones, 2011). Krytenburg *et al*. (1998) observed *Salmonella*

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enterica contamination rates ranging from 5 to 19% in cattle feed in the United States, Europe and South Africa. In a study by Lynn *et al*. (1998), 30% of cattle feed samples from commercial sources and farms in the United States contained *E. coli*; however, none of the tests for *E. coli* O157 were positive. Many investigators recognize that raising poultry on *Salmonella*-free feed can be an important measure in a series of steps to eliminate or minimize the incidence of *Salmonella* in poultry flocks (Schleifer *et al*., 1984).

Individual business operators and regulatory authorities routinely use analysis of animal feed and human food products for microbiological hazards to detect, monitor and control health risks to animals and humans emanating from exposure to these hazards. Information obtained from such analyses can also be used to assess performance of intervention technologies upon targeted microbiological hazards (Jasson *et al*., 2010).

Analyses for microbiological hazards in animal feed can be used to select suitable operating conditions during extrusion cooking to eliminate or minimize the incidence of mesophilic and thermotolerant pathogens in animal feed (Okelo *et al*., 2008). Prevention of food-borne illness requires establishment of control measures throughout the food supply chain: at the production stage, measures to prevent introduction of microbiological hazards (pathogens) to crops and food animals, minimizing multiplication of these pathogens during harvest and post-harvest operations, and additional measures to inhibit or destroy the organisms during processing and use of food (Baird-Parker, 2000).

Microbiological safety of animal feed and feed ingredients can be assured through application of interventions intended to keep feed products free from food-borne pathogens at pertinent points throughout the farm-to-fork continuum. To assess effectiveness of such interventions the feed industry relies primarily on technologies for the detection and enumeration of microbiological feed hazards.

4.2 Microbiological analysis overview

Microbiological analyses comprise methods for detection (largely qualitative methods) and methods for enumeration (quantitative methods) of microorganisms. Qualitative methods for microbiological analyses are those that detect either the presence or absence of organisms, either directly or indirectly, in a certain quantity of the sampled material. Quantitative methods for the enumeration of microorganisms in food or animal feed products are those that determine the number of cells of a target organism either directly (e.g., bacterial colony count) or indirectly (e.g., through measurements based on growth and metabolic activity of target organisms – color, turbidity, conductivity/impedance, etc., of growth medium) in a specified quantity of the sample. Each quantitative method for enumeration

of microorganisms in food and animal feed products has associated lower and upper limits of detection, a parameter that indicates the smallest and largest number of organisms in a specified quantity of sample that can be reliably determined.

Reference methods for microbiological analyses are commonly developed through collaborative laboratory studies, have undergone peer review by independent investigators, and have proven performance. Further, such reference methods for microbiological analysis are generally open access rather than proprietary, widely accepted and often internationally recognized. Regulatory bodies generally recommend the use of these conventionally accepted reference methods for official analysis, although alternative validated methods, which often are proprietary, may be used if they provide equivalent results. Conventional and proprietary methods for microbiological analysis are commonly used in the food industry to detect, identify, and enumerate microbiological hazards as well as to evaluate, monitor, and control the performance of interventions to these hazards.

Microbiological culture techniques for detection and enumeration are based on multiplication of target organisms at appropriate incubation conditions and periods in broth or agar media until their presence becomes evident without the aid of a microscope. The presence of organisms is manifested in the form of changes in turbidity or color or as visible colonies. Microbiological culture techniques have been in existence longer than other microbial analysis methods and are therefore the reference methods in many circumstances. Methods that are based on other principles such as molecular microbiology may be broadly recognized by many analytical laboratories of the food industry and regulatory agencies if they provide a more accurate result than the reference method (Jasson *et al.*, 2010).

4.3 Role and application of culture methods for the detection and enumeration of microbiological hazards

Culture-based methods for microbiological analyses involve multiple steps including media preparation, inoculation, possibly serial dilution, incubation, and colony counting when the purpose is quantification. The fundamental aspect of microorganisms which makes this methodology work is the multiplication of target microorganisms that have been inoculated onto appropriate growth media and incubated under suitable conditions and durations for those organisms.

4.3.1 Detection

In broth, microbiological growth may be observed as color, conductivity, impedance or turbidity changes of the broth media. In agar media, on the other hand, visible colonies of cells indicate microbiological growth.

Appearance of the indicators of growth such as the ones described above is sufficient to conclude presence of viable microbiological hazards in animal feed products. To detect the presence of sub-lethally injured microorganisms, it may be necessary to use a series of culture enrichment steps. For example, in the case of *Salmonella*, enrichment consists of the following procedures (Blackburn, 1999):

1. Pre-enrichment for 16–26 h to allow for resuscitation and multiplication of sub-lethally injured *Salmonella* cells.
2. Selective enrichment for 22–52 h to increase the proportion of salmonellae relative to background microflora.
3. Spread-plating on selective/differential agar media and incubating at appropriate temperatures for 22–48 h to allow appearance of *Salmonella* colonies while inhibiting the growth of other organisms.
4. Confirmation of presumptive-positive *Salmonella* colonies by biochemical and serological analyses.

4.3.2 Enumeration

If the goal of the analysis is to also determine the level of microbiological hazards in the animal feed product, the levels of indicators of growth of microorganisms grown in multiple dilutions of media are measured and a previously determined association curve is used to determine the original hazard concentration in the feed product represented by the sample. For example, the plate count method for the enumeration of bacteria involves determining the total number of bacteria in a food or animal feed product by inoculating dilutions of suspensions of the product onto the surface of a solid growth medium using the spread-plate technique or by mixing the suspensions with specified quantities of molten growth medium in petri dishes. For aerobic and anaerobic or obligate anaerobic organisms, bacterial colonies are counted following incubation under controlled environmental conditions corresponding to the target organism for specified durations and temperatures depending on their characteristics (Jasson *et al*., 2010). Okelo *et al*. (2008) described a method for sample preparation and enumeration for spores of *Bacillus stearothermophilus* ATCC 12980 in an animal feed product in which they reported limits of detection ranging from 1.3×10^2 colony forming units (cfu)/20 g feed to 1.3×10^3 cfu/20 g feed. Jasson *et al*. (2010) reported a quantification limit of approximately 4 cfu/ml for liquid foods, or approximately 40 cfu/g for solid foods based on using 1 ml of primary suspension for surface plating. In a review of quantitative data on food-borne microbiological hazards, Marlony *et al*. (2008) reported that conventional, culture-based methods were the predominant means used for enumerating *Salmonella* in the food and animal feed studies. Conventional, culture-based methods that utilize selective nutritious broth or agar media to grow, isolate or enumerate the target organism while suppressing the growth of background flora of the food matrix are acknowledged as the

reference analytical methods for official control by regulatory authorities; they are regarded as the 'gold standard' in food diagnostics and in international trade and compliance testing. Further, if low numbers (less than 50 cfu/g food or feed) of bacteria are suspected in food or animal feed samples, the most probable number method (MPN) for enumeration may be employed (Jasson *et al.*, 2010). In MPN each of three serial dilutions of samples prepared in the same way as the plate count method is transferred in triplicate into 9 or 15 test tubes containing appropriate liquid medium for the three- or five-tube method, respectively.

Automation of culture-based enumeration methods can reduce the time needed to perform these analytical procedures. Advances in automation of these methods have enabled reduction in manpower needs, material cost, and improved throughput (Marlony *et al.*, 2008). Agar preparation machines, automated dilutors, counting devices, and spiral plate devices for spread-plating are a few examples of equipment that have made it possible for laboratories to process more samples with greater efficiency (Jasson *et al.*, 2010).

4.4 Role and application of molecular methods for the detection and enumeration of microbiological hazards

Genetically based methods for the detection, enumeration and characterization of pathogens in foods utilize hybridization of target DNA with a specific DNA probe (deBoer and Beumer, 1999). Maciorowski *et al.* (2001) detected and characterized male specific or somatic bacteriophages as indicators of fecal contamination, based on their RNA or DNA content, in various types of animal feed with varying levels of bacterial contamination. Although they are asexual, bacteria with pilus or pili are often referred to as 'male'. A male-specific phage infects bacteria via the bacteria's pilus or pili. With *E. coli* as the host, the authors tested a wide variety of feed ingredients and commercial dietary mixes and detected phages in at least one sample of every feed matrix. However, they could not establish a clear association between the incidence of phages and fecal coliforms or *Salmonella* spp. Techniques that employ nucleic acid-based amplification, of which the polymerase chain reaction (PCR) has been the most extensively used, offer numerous advantages over conventional methods such as a shorter time-to-result, low detection limits, greater specificity and increased potential for automation (Germini *et al.*, 2009; deBoer and Beumer, 1999). Analyses using cultural methods for detection and enumeration of microbiological hazards in animal feed can take up to 7 days. Because molecular methods of analysis can take between 24 and 36 hours, a feed mill that produces a few tons of animal feed per hour can thereby monitor the microbiological quality of its feed more efficiently (Maciorowski *et al.*, 2007).

Although procedures for use of PCR in food analyses are rather complex and require relatively clean environments, great advances have been made

in overcoming these obstacles. Other barriers include the following. PCR-based methods cannot distinguish between dead and live cells of microorganisms, the presence of polymerase inhibitors in the food matrix can generate false negatives, and accessibility of target organisms can be a limiting factor. Most of these difficulties are overcome by performing pre-enrichment prior to PCR analyses (deBoer and Beumer, 1999). Molecular-based techniques are especially suited for the detection and enumeration of viable but nonculturable (VBNC) cells, since these techniques do not rely on actively growing cells (Colwell, 1997). VBNC are bacterial cells that are not dead but are not able to grow on routine media unless resuscitated from their VBNC state.

4.5 Role and application of emerging technologies for the detection and enumeration of microbiological hazards

4.5.1 Chromogenic and fluorogenic growth media

Certain growth media are formulated to contain enzyme substrates which, when hydrolyzed by specific enzymes of target organisms, produce unique pigmented (chromogens) or fluorescent (fluorogens) substances that in turn manifest as color changes or fluorescence in the growth media. Detection is based on direct spectrophotometric measurement of absorption by chromogens or fluorescence by fluorogens when illuminated by ultraviolet light at a defined wavelength. Incorporation of such enzyme substrates into selective and differential growth media enables the identification and enumeration of certain microorganisms. (Manafi, 1996; Jasson *et al*., 2010).

Chromogens and fluorogens are either absorbed into the cells of microorganisms, thereby specifically labeling them, or they diffuse into the growth medium giving a pronounced color change or fluorescence. Chromogenic and fluorogenic media are commonly used to detect and enumerate important indicator organisms in food and water. Briefly, Kilian and Bulow (1976) and Kilian and Bulow (1979) reported that approximately 97% of *E. coli* strains but only a few strains of *Salmonella, Shigella* and *Yersinia* produce β-D-glucuronidase (GUD), an enzyme that catalyzes the hydrolysis of β-D-glucopyranosiduronic acids into their corresponding aglycons and D-glucuronic acid. Hartman (1989), Manafi and Rotter (1991) and Frampton and Restaino (1993) also reported that between 96 and 98% of *E. coli* strains produce GUD. Further, certain pathogenic strains of *E. coli* such as *E. coli* O157:H7 do not produce GUD (Chang *et al*., 1989; Thompson *et al*., 1990). Rice *et al*. (1991) also reported that some strains of *E. coli* spp. do not produce GUD. Some strains of other bacteria such as flavobacteria (Petzel and Hartman, 1986), staphylococci (Moberg, 1985), streptococci (Rod *et al*., 1974) and clostridia (Sakaguchi and Murata, 1983) have also been shown to produce GUD. GUD interacts with various enzyme

substrates present in the chromo- and fluorogenic media to produce specific but distinct colors (yellow, red, pink, blue) and fluorescence (Manafi, 1996). By careful selection of incubation conditions and incorporation of selective agents in the media that only permit the growth of *E. coli* and coliforms, chromogenic and fluorogenic media may be used to identify and enumerate these organisms in food and water. Similarly, Manafi (1996) has described these culture media for the rapid detection of salmonellae, clostridia, *Streptococcus pyogenes, Staphylococcus aureus,* and enterococci that utilize the phenotypic characteristics of these organisms to identify and enumerate them. Similar culture media with additional capability for distinguishing Gram-positive from Gram-negative bacteria based on the L-alanine-aminopeptidase activity of the Gram-negative bacteria have been developed (Manafi and Kneifel, 1990). The use of chromogenic and fluorogenic growth media for the detection and enumeration of microorganisms can eliminate the need for subculture and associated biochemical testing, thereby saving on supplies and time requirements (Manafi, 1996).

4.5.2 Impedance-based techniques

Actively growing bacteria produce charged end-products that alter the impedance of their growth culture medium. Because the change in impedance observed is proportional to the number of bacteria in the culture medium, a predetermined mathematical relationship can be used to detect the presence or absence of target organisms or to estimate the number of microorganisms in a specified quantity of the sample of food or animal feed product.

Impedance-based assays have been developed for the detection and enumeration of *Salmonella*, *Listeria* and *Campylobacter* (Gibson *et al.*, 1992). These systems can be used to examine hundreds of separate samples simultaneously, since they are amenable to automation and control using computers. Analysis of food samples for contamination with microorganisms can be completed in 24 hours (deBoer and Beumer, 1999). Because they save time and supplies, these techniques are typically used to estimate total bacterial counts and for screening large numbers of food samples (van der Zee and Huis in 't Veld, 1997). deBoer and Beumer (1999) found that impedance-based techniques were not suited for testing food samples with low numbers of microorganisms. An additional drawback is that the properties of the food matrix may influence impedance of the samples, thereby necessitating the determination of calibration curves for each food matrix examined.

4.5.3 Bacteriophage-based techniques

Bacteriophages are obligate intracellular bacterial parasites that grow rapidly and are extremely host-specific, able to infect specific species or

even strains, with a few exceptions. The highly specific interaction of phage with its bacterial host has been used to develop assays for the detection of foodborne pathogens. For example, in an assay for *Salmonella*, a specific bacteriophage was engineered to carry a detectable marker. In the presence of *Salmonella*, the phage confers the marker to the host, which then expresses the phenotype to allow the detection of the *Salmonella* (Tenover, 1988). Detection of pathogens using phages allows the distinction between living and dead bacterial cells and is much more rapid than conventional culture techniques. Phages can be used to detect the presence of pathogens in food or the food production environments, thereby allowing quick and specific identification of viable cells (Hagens and Loessner, 2007).

4.6 Future trends

As advances in 'real-time' microbiology continue, it may be expected that detection and enumeration that are based on all of the existing methods will become more amenable to automation and better adapted to computer technology to control processes, analyses, and the reporting of results. Real-time microbiological detection and enumeration results for target organisms can be used as input data to control mechanisms that regulate critical operating conditions of process equipment during the manufacture of animal feed products. When feed-borne pathogens are monitored and controlled during processing in this manner following prescribed HACCP protocols or similar food safety principles, consistent microbiological quality can be achieved. Prevention, elimination, or reduction of microbiological contamination of animal feed products is a multifaceted undertaking that will continue to rely on advances in detection and enumeration techniques to monitor and control feedborne pathogens with a view to assuring a safe supply of feed for animals.

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5

Assessment of the microbiological risks in feedingstuffs for food-producing animals

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Abstract: This chapter describes a qualitative microbiological risk assessment (MRA) in feedingstuffs in the EU. It identifies *Salmonella* spp. as the major hazard, and oil seed meal and animal-derived protein as the major risk feed materials. The transmission of *Salmonella* from feed to animals, and to derived food products, has already been established, but there have also been some reports which establish a direct link between contaminated feed, contaminated food and human cases of salmonellosis. Feed manufacturers should be aiming to constantly reduce the occurrence of *Salmonella*. HACCP, GHP and GMP protocols are valuable tools for improving food and feed safety and should be implemented throughout the entire production line, starting with the raw feed materials. The establishment of microbiological criteria (process hygiene criteria at critical stages) for *Salmonella* contamination along the feed chain is suggested as one of several control tools.

Key words: feed, *Salmonella*, *Listeria*, *Clostridium*, *Campylobacter*, microbiological criteria, risk assessment.

5.1 Introduction

The feeding of food-producing animals is complex due to the diversity of species and production systems. The increase in animal production in the European Union (EU) has been followed by an increase of the amount of feedingstuffs produced, the main type being compound feedingstuffs. The scope of this chapter is to give an overview of microbiological risk assessment (MRA), for both animal and public health, due to the presence of pathogenic bacteria in feedingstuffs, and to discuss the most important options to control the risk. The BIOHAZ Panel and its scientific secretariat provide advice on questions on biological hazards relating to food safety

and food-borne disease, including food-borne zoonoses and transmissible spongiform encephalopathies, microbiology, food hygiene and associated waste management. Recently, the impact of the microbial contamination of feedingstuffs on *Salmonella* prevalence in animal populations, food and humans has been assessed by the BIOHAZ Panel (http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902004131.htm). The information presented in this chapter largely derives from the BIOHAZ opinion.

5.1.1 Legislative framework for the safety of feedingstuffs in the EU

In the EU, Regulation (EC) No. 183/2005 on feed hygiene lays down obligations for feed business operators. In accordance with Article 5(3), specific microbiological criteria and targets shall be adopted. Certain microbiological criteria for feedingstuffs are set in the national legislation of some Member States. However, these criteria are not harmonized in the EU for the majority of feedingstuffs, the only exceptions being maximum contents and guidance values for mycotoxins and requirements for animal by-products that can be used as feed materials and pet food, which are laid down in Regulation (EC) No. 1774/2002 concerning animal by-products not intended for human consumption.

Feedingstuffs comprise several types of substances and products used for feeding of animals: feed materials (list is laid down in Council Directive 96/25/EC), feed additives, pre-mixtures and compound feedingstuffs.

The European Commission is considering the establishment of specific microbiological criteria and targets for feedingstuffs for food-producing animals in Community legislation on feed hygiene. These criteria will take into account the impact on public health and on animal health.

5.1.2 International activities in the field of MRA in feedingstuffs

Various international bodies have addressed feed and food safety requirements. The main objectives and conclusions of these assessments can be summarized as follows.

- *FAO, 1998*. Animal Feeding and Food Safety. Report of an FAO Expert Consultation, Rome, FAO Food and Nutrition Paper No. 69 (http://www.fao.org/docrep/w8901e/w8901e00.htm). Conclusions: certain biological agents (specific mention of TSE, *Salmonella*, *Toxoplasma* and *Trichinella*) incorporated into feed, either intentionally or unintentionally, can result in hazards in food of animal origin and may enter feed at any stage of production up to the point of feeding. Where foodborne hazards originate in feed, the hazard should be adequately controlled.
- *Codex Alimentarius Commission (FAO/WHO), 2004*. Code of Practice on Good Animal Feeding (CAC/RCP 54-2004, http://www.codexalimentarius.net/download/standards/10080/CXP_054e.pdf).

Objective: to help ensure the safety of food for human consumption through adherence to good animal feeding practice at the farm, and good manufacturing practices (GMPs) during the procurement, handling, storage, processing and distribution of feed and feed ingredients. Conclusions: all feed and feed ingredients should meet minimum safety standards. It is essential that levels of undesirable substances (including pathogenic agents) are sufficiently low in feed and feed ingredients that their concentration in food for human consumption is consistently below the level of concern.

- The issue of re-establishing an ad hoc *Codex intergovernmental task force on animal feeding* is being currently considered after circulation of a letter for proposals (ftp://ftp.fao.org/codex/Circular_letters/CXCL2007/cl07_19e.pdf) for future work by Codex on animal feeding.
- The development of a *horizontal OIE standard on animal feeding* commenced in 2006. Under the guidance of the Terrestrial Animal Health Standards Commission and the Aquatic Animal Health Standards Commission, the OIE is currently developing standards on animal feeding, with the primary objective of preventing disease transmission and spread via feed. Chapter 6.3 of the *Terrestrial Animal Health Code 2009* (http://www.oie.int/eng/normes/MCODE/en_chapitre_1.6.3.htm) deals with the control of hazards of animal health and public health importance in animal feed. This chapter provides guidance on animal feeding in relation to animal health.
- *FAO/WHO, 2007*. Animal Feed Impact on Food Safety (ftp://ftp.fao.org/docrep/fao/010/a1507e/a1507e00.pdf). Objective: to review current knowledge on animal feed and its impact on food safety and international food and feed trade. In the area of microbiological hazards, the document concluded that the primary sources are contaminated pastureland, forages and animal and vegetable protein fed directly to animals. The group reviewed a number of hazards but was unable to rank them.

5.2 Hazard identification

This chapter does not contemplate hazards such as BSE/TSE, presence of agents such as parasites and viruses, and contamination by fungi and mycotoxins.

5.2.1 *Salmonella* spp.

Subtyping of isolates of *Salmonella* into serotypes is an important tool for tracing the sources to *Salmonella* contamination of the feed and food chain, and of subsequent infections of animals and humans. Several studies have revealed the substantial risk for the feed production to be exposed to a great diversity of serotypes (Rutqvist and Thal, 1958; Karlsson *et al*., 1963;

Hurvell *et al*., 1969; Gunnarsson *et al*., 1971; Sandstedt *et al*., 1980; Martensson *et al*., 1984; Eld *et al*., 1991; Malmqvist *et al*., 1995; EFSA, 2009).

The isolation of *S.* Enteritidis occurs only occasionally, except perhaps in Japan (Shirota *et al*., 2000), whilst *S.* Typhimurium appears to be a more widespread contaminant, albeit not a dominant one (EFSA, 2006a, 2007a; Anon., 2006b; Malmqvist *et al*., 1995). In the monitoring of *Salmonella* in the animal feed sector (Anon., 2007a) five *Salmonella* serotypes (Enteritidis, Typhimurium, Infantis, Virchow and Hadar) are classified as critical. During 2006 the frequency of isolation of these critical serotypes was as follows for different feed materials: South American soya meal and rape seed meal: both 20%; soya beans toasted: 11%; European sunflower meal: 50%; fish meal, egg shells and French wheat bran: all 0%.

The repeated and long-term isolation of certain serotypes in feed ingredients or compounded feed has often been found to be the result of persistent contamination of crushing and feed producing plants. Although only a minority of the serotypes isolated from animal feed are found to cause clinical disorders in animals, they may all be pathogenic to humans. Contamination of feed with serotypes pathogenic or adapted to certain animal species (e.g. *S.* Typhimurium and *S.* Enteritidis) usually results in intestinal colonization and long-term shedding, which favours persistence of the infection at the farm level, with the additional risk for further spread of the infection to other animal holdings, to the environment and to humans.

Salmonella infection of livestock has differing manifestations according to the livestock species and serotype(s) involved. The evidence of surveillance and of clinical experience is that infections of pigs and poultry are often widespread in many EU Member States but typically asymptomatic, whilst ruminants may be less frequently infected but more often show clinical signs. Certain *Salmonella* serotypes are evidently species-adapted and associated with predictable clinical disease, relevant examples being *S.* Gallinarum and *S.* Pullorum (causing fowl typhoid and pullorum disease respectively in poultry), *S.* Choleraesuis (causing enteritis and septicaemia in pigs), *S.* Abortusovis (causing abortion in sheep) and *S.* Dublin, associated with abortion, enteritis and septicaemia in cattle. Among these, *S.* Dublin remains widespread in European livestock production. Data on prevalence of *Salmonella* contamination in the animal production varies by animal species, country and detection methods applied (EFSA, 2009).

5.2.2 *Listeria monocytogenes*

Listeria monocytogenes is common in soil, sewage, forage and water and consequently can be present in different vegetation. *Listeria* spp. have been found in poultry feeds both before and after the heat treatment (Blank *et al*., 1996; Whyte *et al*., 2003). In a study (Whyte *et al*., 2003) it was noted that much of the environment in the feed mill was contaminated with *Listeria* spp. which could suggest that recontamination of pelleted feed may occur.

The prevalence of *L. monocytogenes* in animal feed having a low level of available water (a_w) such as hay and cereal grains is very low and the numbers are probably unlikely to reach levels that present a serious risk to animals (Fenlon, 1999). Wet feeding of pigs during the fattening period was identified as a risk factor for *L. monocytogenes* (Beloeil *et al*., 2003). Furthermore, it is well known that the risk of the presence of *L. monocytogenes* in silage is related to animal listeriosis and asymptomatic carriage, mainly in dairy cattle, sheep and goats (Nightingale *et al*., 2004; Skovgaard and Morgen, 1988; Wagner *et al*., 2005). The link between aerobically spoiled silage and cases of listeriosis in farm animals has been described; in addition the risk of the presence of *L. monocytogenes* in raw milk is higher when cows are fed with silage with pH 4 and above.

5.2.3 *Escherichia coli* O157

Escherichia coli O157:H7 has rarely been detected in cattle feed. However, recent reports suggest that feed may be a source of *E. coli* O157 in cattle (Dodd *et al*., 2003; Sargeant *et al*., 2004; Dargatz *et al*., 2005). Hancock *et al*. (2001) reported that 0.5% of purchased feed stored at the farm was positive for *E. coli* O157. Reports have also shown that *E. coli* O157 may multiply in some cattle feeds where there is sufficient water content (Lynn *et al*., 1998; Fenlon and Wilson, 2000). In a recent paper, it is concluded that the time/temperature combinations used in commercial pelleting processes do not effectively kill high numbers of *E. coli* O157 present in the feed (Hutchinson *et al*., 2007).

5.2.4 *Clostridium* spp.

Clostridium perfringens is an obligate spore-forming anaerobe, common in faeces (Tschirdewahn *et al*., 1991) and soil (del Mar Gamboa *et al*., 2005). It is therefore a common component of feedstuffs, as either vegetative cells or endospores (Xylouri, 1997) and is particularly prevalent in soil-contaminated feeds such as root crops (Secasiu, 1982). Among feedstuffs, animal protein sources and compounded feeds usually have the higher frequency of contamination (Chakrabarty and Boro, 1981; Kaić, 1977; Xylouri, 1997), with a higher concentration of *C. perfringens* (Wojdat, 2006) and a higher prevalence of toxigenic strains (Secasiu, 1982; Wojdat, 2006). Prió *et al*. (Prió, 2001) reported that there was little or no correlation between levels of clostridial contamination in raw ingredients and in compounded feeds derived from them.

In view of the common isolation of *C. perfringens* from the environment and from the intestinal tracts of livestock (75–95% of broilers) (van Immerseel, 2004), and the fact that *C. perfringens*-associated diseases appear to need initiators in addition to the presence of the organism (Craven, 2000; Songer, 1996; van Immerseel, 2004), the significance of feed contamination

by this bacterium is open to question. Feed has, however, been implicated in some fowl necrotic enteritis outbreaks (Dosoky, 1990; Frame and Bickford, 1986), and in one study of four pig farms, the unit having toxigenic *C. perfringens* in sow feed had the highest mortality for necrotic enteritis in piglets (Udovičić, 1994).

Most clinical cases of *Clostridium botulinum* intoxications are related to equines and cattle (Galey *et al*., 2000; Kelch *et al*., 2000). The common source of the toxins is silage or haylage of poor quality. Spreading onto pasture of contaminated poultry litter containing sometimes dead poultry can be a source of contamination of cattle (Anon., 2002). Although the source can sometimes be difficult to find, in general the cause of botulism in animals is the multiplication of and the toxin production by *C. botulinum* in the feed consumed. As an example, in France, *C. botulinum* type A (Gimenez and Ciccarelli, 1987), type C (Dohms *et al*., 1982) and type D (Popoff, 1989) were present in feed sampled at the farm where outbreaks occurred. Nevertheless, detection of the presence of the toxin in the feed is often very difficult (Anon., 2002).

5.2.5 *Campylobacter* spp.

No published data were found in the literature search indicating that commercial feed is a source of *Campylobacter* infection in food-producing animals and this is unlikely because of the dry conditions and exposure to air involved in feed production.

5.2.6 Antimicrobial resistance in bacterial contaminants of feedstuffs

There appears to be ample potential for the introduction of antimicrobial-resistant bacteria to animal production units by feedstuffs, given the limitations of conventional feedmill treatments in eliminating common bacterial contaminants (da Costa *et al*., 2007). The subsequent risk of increasing resistance on-farm and beyond relates not only to successful colonization by resistant strains of feed origin, but also to the potential for the dissemination of mobile resistance elements to other bacteria. Integron sequences, with the capacity to accept and transmit antimicrobial resistance gene cassettes (Hall and Collis, 1998), are commonly found amongst Gram-negative bacteria isolated from rendered animal feedstuffs, in association with widespread antimicrobial resistances (Hofacre *et al*., 2001). A particular concern is the potential for international dissemination in feed or ingredients of plasmids carrying a variety of genes which confer resistance (e.g. to third- and fourth-generation cephalosporins).

5.2.7 Conclusion (main hazards)

According to the reviewed literature, the most important bacterial pathogen in feed is *Salmonella* which frequently occurs in a large number of feed

ingredients and also in compounded feed. Data from studies of *Salmonella* prevalence in forage, home-grown cereals and purchased straight feedingstuffs are scarce.

Other pathogenic bacteria with relevance for animal and human health and where feed might be a vector for the dissemination of the pathogen are limited to a few other species. The problems with *Listeria* seem to be primarily limited to the occurrence in silage of poor quality. Feed has been suggested to be a source of infection with *E. coli* O157:H7 in cattle; however, no information is available on whether these feed strains cause human disease. *Clostridium botulinum* present in poor-quality silage may cause serious intoxications in equines or bovines, and *C. perfringens* is commonly isolated from several feed ingredients.

All following considerations of this paper are focused on the main microbiological hazard (*Salmonella*) and on industrial compounded feed, including its major risk ingredients: protein-rich vegetable protein and animal-derived protein.

5.3 Exposure assessment

5.3.1 Feed production and consumption (including imports) in the EU27

The information in this section is from FEFAC (the European Feed Manufacturers' Federation, http://www.fefac.org) and FEDIOL (the EU Federation of the European Bean Crushers, Protein Meals Producers and Vegetable Oil Producers, oil and protein meal industry, http://www.fediol.be/). Information on the number of animals by production categories (animal species) in the EU27 can be found on 'Eurostat 2007' available at http://epp.eurostat.ec.europa.eu. Hence the data summarized in the following section reflect the situation in 2007.

Total consumption of feed by the major food animal species in EU27

About 470 million tonnes of feedstuffs are fed to the major food animal species. Of these, about 49% is forage, 30% industrial compound feed (141.7 million tonnes), 12% home-grown cereals, and 9% purchased straight feedstuffs (purchased feed materials intended for direct feeding on farm).

Compounded feed production by the EU27 feed industry

A total of 141.7 million tonnes of compounded feed is produced, out of which about 34% is used for pigs, 31% for poultry, 27% for cattle, 7% for other species and 1% for use as milk replacers. The major feed materials used (percent of total) are 47% feed cereals, 27% oil seed residues (cakes and meals), 13% co-products from the food industry, 3% minerals, additives, and vitamins, and several other minor categories such as dried forage, dairy products, oils and fats, pulses, tapioca and others.

The production and use of oil seed meals in the EU27
About 40 million tonnes of oil seed were crushed in 2006. The dominating sources of seed are soy beans and rapeseed. Approximately 150 production units operate across the EU. The majority (80%) of the oil seed producers ('crushers') are organized in FEDIOL, with a total crushing capacity of about 30 million tonnes.

Imports of feedstuffs and feed ingredients
The contribution of imports to the total amount of compound feedstuffs used in the EU varies with the type of components. Of the 278 million tonnes of cereals consumed in the EU, only 16 million tonnes are imported. The EU is currently dependent on importing vegetable protein. According to FEFAC, self-sufficiency in the EU25 is 2% for soy bean meal but significantly higher (72%) for rapeseed meal. The imported oil seed meal is produced in crushing plants in the exporting countries, mainly in Argentina and Brazil.

5.3.2 Prevalence of *Salmonella* in feed materials

Most ingredients of both animal and plant origin used in compound feed seem to be prone to *Salmonella* contamination. Ingredients used for animal feedingstuffs have been shown to be commonly contaminated with *Salmonella* (EFSA, 2006a, 2007a; Anon., 2006b, 2007a; Dargatz *et al*., 2005; Hacking *et al*., 1978; Jones and Richardson, 2004; Kidd *et al*., 2002). However, prevalence data for *Salmonella* in feed ingredients or compounded feed are usually very difficult to compare between different studies, due to differences in sampling and analytical methods applied. *Salmonella*, when present in feed, is usually in low numbers and is unevenly distributed, which makes the sampling critical, and raises questions about the confidence in negative results. Some studies presenting data on the prevalence of *Salmonella* in different feed ingredients are summarized here.

Animal-derived protein
To prevent the spread of BSE, a total ban on feeding processed animal protein in feeds for any animal farmed for the production of food was introduced on 1 January 2001. Since then, some exceptions have been introduced, such as the use of fish meal and certain blood products and dicalcium phosphate as feed for non-ruminants, as described in the current legislation (EC No. 1292/2005, http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_205/l_20520050806en00030011.pdf).

When allowed as ingredients of animal feed, mammalian meat and bone meal (MBM) and poultry offal meal were found to be frequently contaminated by *Salmonella*, a logical consequence of the risk from the rendering of animals infected with *Salmonella* (Thal *et al*., 1957; Hirsch and Sapiro-Hirsch, 1958; Knox *et al*., 1963). Fish meal also has been historically found

to be contaminated by *Salmonella*, according to the EFSA's zoonoses reports (EFSA, 2006a, 2007a). In 2009, a marked decrease in contamination was observed (0.7% in 2009 compared to 2.1% in 2008) (EFSA, 2011). Contaminated fish meal was the source of the most well-known example of feed-borne transmission of *Salmonella*, when *S.* Agona emerged as a public health problem in several countries (Clark *et al*., 1973; Crump *et al*., 2002).

There is also a potential risk for the spread of *Salmonella* by feeding some dairy by-products to animals (EFSA, 2006b). In summary, animal-derived protein is considered as a high-risk product for *Salmonella*. However, that risk is currently limited in the EU, though it may exist in third world countries and recur in the EU if in the future such products are again allowed as animal feed.

Vegetable protein: non-processed products

Data from non-processed products are scarce. *Salmonella* were isolated from approximately 30% (12–68%) of samples tested from dust of all lots of soy beans imported mainly from South America to Norway during 1994–2007 (Denofa, 2007).

Vegetable protein: processed products

Several studies illustrate that products from the crushing industry are often contaminated with *Salmonella*. In a Dutch report, 3.2% and 6.7% of feed materials (Brazilian-extracted soy beans) from 2002 and 2003 respectively, were positive for *Salmonella* (Anon., 2004). In Sweden 14.6% of imported consignments of soy meal were found to be contaminated by *Salmonella* during 2004–2005, and when considering only imports mostly from South America the level was approximately doubled (Wierup, 2006). That level has regularly been found in the Swedish feed control where all consignments are tested before introduction to the feed mills (Häggblom, 1994). A significantly higher prevalence of *Salmonella* was found in Sweden in feed mills using contaminated soy meal in contrast to those supplied by a safer source (Wierup, 2006). Denmark also reports problems associated with *Salmonella*-contaminated vegetable protein, primarily imported soy meal (http://www.pdir.dk). Available data from the EFSA zoonoses reports also support oil seeds, e.g. soy bean products, as a risk factor for introducing *Salmonella* into the feed chain (EFSA, 2006a, 2007a, 2011).

Salmonella is also frequently reported from rape seed and palm kernel. Product Board Animal Feed (Anon., 2007a) reports a contamination rate for rape seed meal and flakes of 6.8% and 3.4% for the years 2005 and 2006 respectively (number of samples: 4378 in 2005 and 4337 in 2006). In a Dutch monitoring study, 12% of extracted rape seed meal were positive in 2002, and 7% in 2003 (Anon., 2004). In a Swedish study 10% of rape meal batches and 9% of cornmeal batches were found to be *Salmonella* contaminated (Wierup, 2006). In a recently published study, *Salmonella* was frequently isolated from consignments of soybean meal and rape seed meal imported

to Sweden (Wierup and Häggblom, 2010). UK data record an improving trend in the contamination of oilseed meals and products from 3.3% in 1999 to 1.7% in 2006 (Anon., 2007b).

Grain

Grain is seldom found to be contaminated unless as a result of contamination during storage and transport. However, studies show that grain can also be contaminated, especially if it originates from areas where *Salmonella* is common in wildlife or local livestock. It is common to find *Salmonella* serotypes which are associated with wildlife or local livestock in dust collected from grain drying and handling systems and grain storage bins in compound feed mills (Davies and Wray, 1997). From the zoonoses reports from 2005 and 2006, cereals generally seem to be less contaminated than processed animal or plant protein (EFSA, 2006a, 2007a). UK data suggest a 0.3% contamination rate of grain in 2006 (Anon., 2007b). However, it is possible that the contamination of grain may be underestimated because of the relatively small surface area of the material tested (Jones and Ricke, 1994) compared with vegetable oil-seed residue meals.

Forage

Data on *Salmonella* contamination of forage seem to be very scarce but generally forage feed is not associated with a risk of contamination with *Salmonella* unless in exceptional cases.

5.3.3 Prevalence of *Salmonella* in compound feed

Contamination of compounded feed by *Salmonella* is not uncommon even in feed that has undergone heat treatment (Cox *et al.*, 1983; Hacking *et al.*, 1978; Österberg *et al.*, 2006; Veldman *et al.*, 1995). Recent data from EU Member States (MS) show a national prevalence for compounded poultry feed of 6% in one MS, while most other countries have prevalences in the range from 0% to 1.5% (EFSA, 2006a). Similar contamination rates were reported for pig (up to 1.7%) and cattle (up to 2.4%) feeds in the EU. In MS with a low prevalence of *Salmonella* in food-producing animals, *Salmonella* is only occasionally found in compounded feed. The industry-based data from 2005 and 2006 (Anon., 2007a) report an incidence between 0 and 0.8% of *Salmonella*-contaminated samples in compounded feed to different food animal species (poultry, swine and cattle). The lowest prevalence was found in feed for top breeding poultry flocks and the highest for laying hens. UK data from 2006 report a 0.4% contamination rate of pelleted poultry feed and 0.6% for pig and poultry meals (Anon., 2007b). Incidence of *Salmonella* in feed has been studied in a Spanish surveillance programme in feed developed during 2007. A total of 700 feed mills were visited, with 2100 feed materials and 2100 compound feed batches sampled. Preliminary results from 308 feed mills showed a 3.5% incidence in feed materials and

3.5% incidence in compound feed (for all *Salmonella* serotypes) (Sobrino, 2008).

Surveys of the prevalence of *Salmonella* in feed mills have shown that *Salmonella* was frequently recovered from the pre-heating as well as from the post-heating treatment areas of the mill. A high *Salmonella* prevalence was also detected in dust samples from the post-heating treatment area of the mill and in feed delivery vehicles, as well as inside the pellet cooling systems (Davies and Wray, 1997; Whyte *et al*., 2003).

5.4 Considerations on sampling and detection methods for *Salmonella* in feed

5.4.1 Sampling for *Salmonella* in feed and design of sampling plans

The uncertainty in sampling is largely dependent on how the contaminant is distributed in the lot. The more unevenly distributed the contaminant is, the more samples are needed to obtain the same level of confidence. It is recognized that a large number of samples must be taken to increase confidence in negative results (McChesney *et al*., 1995).

Despite the relatively large numbers of publications reporting figures for the prevalence of *Salmonella* in raw materials and feed, there are essentially no data regarding the uncertainty of the sampling procedure. Mechanical (automatic) sampling for *Salmonella* is the method most often used. The heterogeneous distribution of *Salmonella* in feed materials requires sampling procedures that are adapted for this situation. A large number of small samples are needed to accurately detect *Salmonella* in a lot. Dust and fine particles are more likely to be contaminated with *Salmonella*. Thus the sampling of dust in filters or equipment is a good indicator if *Salmonella* is present in the mill. Sampling according to HACCP (in different parts of the processing line) is more sensitive and cost-effective than testing of the finished product or feed materials.

Very little information seems to be available in the scientific literature on sampling plans for *Salmonella*, or quantitative data on occurrence of *Salmonella* in feed or food (Lunestad *et al*., 2006). In a few older studies where most probable number (MPN) techniques were applied, low levels of *Salmonella* were reported (Gunnert and Brest, 1969).

5.4.2 Methods for detection of *Salmonella* in feed

The international standard cultural method for detection of *Salmonella*, ISO 6579, consists of a non-selective pre-enrichment in buffered peptone water, selective enrichment in Rappaport–Vassiliadis (RVS) and Muller–Kaufmann tetrathionate–novobiocin broth (MKTTn) plating on selective solid medium xylose lysine deoxycholat agar (XLD) and another selective medium such as brilliant green agar (BGA) and finally serological and

biochemical confirmation. The ISO6579:2002 (Annex D) MSRV based method has been adopted as the EU standard method for monitoring zoonotic *Salmonella* in samples from primary food animal production. This method also performs well for feed samples but has not yet been formally validated.

Quantitative data for the levels of bacteria are important for developing improved sampling plans. The present methods for isolation of *Salmonella* give qualitative results (absence/presence). The traditional MPN method is labour-intensive and not suitable for large-scale operation; however, simplified methods based on microtitre plates are being developed by ISO/TC34/SC9 (http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=36534).

Immunological and molecular methods for *Salmonella* in feed have been reviewed (Maciorowski *et al*., 2005, 2006). A major obstacle is that *Salmonella* levels are typically low, often less than 1 bacterium/g (D'Aoust and Sewell, 1986). These alternative methods are more rapid and also possible to automate. Drawbacks is cross-reactivity (for immunological methods), and the need for pre-enrichment.

5.4.3 Conclusion on sampling and isolation methods

Salmonella contamination of feed normally involves small numbers of organisms distributed in a non-uniform way within very large consignments of material. The mechanical sampling using grain spears which is normally carried out is often insufficient, and automated in-line sampling devices are preferred to obtain a more representative sample of ingredients and finished products and to increase confidence in negative results. In addition to this, indirect indicators of contamination in the throughput of the mill can be used to assess the likelihood of regular contamination of ingredients or process contamination such as resident *Salmonella* in cooling systems. Similar principles can be applied to oil crushers and feed compounders. Examples of suitable indicator sampling points are dust from intake auger pits, pooled dust from ingredient bins, dust and cleanings from coolers, dust from crumblers and pellet shakers, pooled dust from finished product bins, and dust from outloading gantries. A finding of contamination in these areas should trigger further, more detailed investigation of possible sources.

5.5 Assessment of the contribution of feed as a source of *Salmonella* infections in animals and humans

5.5.1 Assessment of the extent to which feedingstuffs contaminated with *Salmonella* can contribute to the prevalence of infection with *Salmonella* in animals

Several studies have shown strong links between contamination of feedstuffs or feed mills and infections of groups of chickens (Boyer *et al*., 1962;

Davies *et al*., 2001; Shapcott, 1985; Wierup *et al*., 1988), turkeys (Nayak *et al*., 2003; Primm, 1998; Zecha *et al*., 1977), pigs (Davies *et al*., 2004; Newell *et al*., 1959; Österberg *et al*., 2006; Kranker *et al*., 2001) and cattle (Jones *et al*., 1982; Davis *et al*., 2003; Glickman *et al*., 1981) with *Salmonella* of the same serotype. Contaminated haylage (Glickman *et al*., 1981) and vegetable fat (Jones *et al*., 1982) were shown to be the cause for *S.* Anatum or *S.* Mbandaka infections respectively, in cattle. In addition, risk factor analyses and case control studies have incriminated feedmills in poultry and cattle infections (Anderson *et al*., 1997; Chadfield *et al*., 2001). Serotype patterns in animals can suggest feedmill sources (Snow *et al*., 2007), but most strains found in feedstuffs do not become established on farms (Shapcott, 1985; Veldman *et al*., 1995); there is likely to be a strong filtering effect exerted by the endemic farm microflora, animal susceptibilities, feed storage conditions and doubtless many other factors. Pulsed-field gel electrophoresis restriction patterns of DNA have been used to demonstrate that isolates with indistinguishable fingerprints can be isolated from feed and food-producing animals or eggs (Häggblom and Aspan, 1999; Shirota *et al*., 2001).

Salmonella infection with currently prevalent serotypes is rarely seen to cause health problems among chickens, but enteric and other clinical disease is seen more often with turkeys and pigs, and most frequently amongst infected ruminants. Young weaning pigs are commonly affected by *Salmonella* infection in the feed in countries where routine medication is not used, and in some cases the infection causes clinical disease (Sauli *et al*., 2005). Adult pigs have also been shown to be susceptible to infection (Österberg *et al*., 2006; van der Wolf *et al*., 1999). Another study (Berends, 1996) estimated that about 15–30% of all infections in the finishing period may be attributed to contaminated feed. Data from studies on dairy farms suggest that subclinical infection in cattle carried by *Salmonella* serotypes associated with feed is common (Davison *et al*., 2006) and some of these strains may subsequently be involved in human illness (USDA:APHIS:VS, 2005). The risk of acquiring *Salmonella* from feedingstuffs relates to the frequency of contamination and the dose ingested; however, experimental data on infective doses cannot be applied to contaminated feed without additional considerations (Jones *et al*., 1982; Hinton, 1988; Schleifer *et al*., 1984).

Examination of surveillance data indicates that feedstuff *Salmonella* overlap to varying degrees with clinical- or surveillance-derived serotypes from animals. In the UK in 2005, the commonest serotypes isolated from poultry feed (*S.* Livingstone and *S.* Kedougou) were also among the top three isolated from chickens (*S.* Livingstone, *S.* Senftenberg and *S.* Kedougou in rank order). In the same report (Anon., 2006b), *S.* Typhimurium was the only serotype of the relatively few feed isolates to coincide with the top three serotypes reported from cattle, sheep and pigs, respectively. The data from the EU as a whole (EFSA, 2006a, 2007a, b, c) shows more regional variation, as would be expected, and suffers from variability in methodologies and which Member States contribute to each data class. Notably,

however, *S.* Typhimurium is prominent among isolates from chickens, pigs, ruminants and additionally from feedstuffs. Serotypes Infantis and Mbandaka are common amongst broilers, layers and feedstuffs, but conversely it should also be noted that the top three feedstuff serotypes (*S.* Livingstone, *S.* Senftenberg and *S.* Montevideo) are not apparently widespread and common elsewhere, whilst serotypes Enteritidis and Hadar are common amongst layers and broilers but not feedstuffs.

In summary, consideration of individual studies clearly shows the potential of feedstuffs to infect groups of animals with *Salmonellae*, but surveillance data are more equivocal on the matter of their relative importance. Certainly there is overlap between the serotypes commonly found in feed and in livestock, with *S.* Typhimurium being a case in point. Although persistent environmental contamination has been shown to be a major factor in the infection of layer flocks (Davies and Breslin, 2003; Gradel *et al.*, 2004; van de Giessen *et al.*, 1994), contaminated feedstuffs may make a significant contribution to the problem, particularly as layer rations typically are not heat-treated, and may in particular be an important route for the infection of previously uncontaminated henhouses. Similarly, endemic infections on pig premises are likely to be of primary importance, but contaminated or recontaminated feed is considered a significant risk factor and may account for 15–30% of *Salmonella* infections in the finishing period (Berends, 1996). Feed contamination will be of increased importance on units or regions with low prevalence status where endemic infection is well controlled or absent (Shapcott, 1985). For example, in Sweden feed is the major source when *Salmonella* is found to be introduced in particular to swine and poultry meat production (Wierup, 2006). In cases such as all-in/all-out production of broiler and turkey breeding, and production flocks which are operated to a high standard of biosecurity, feed becomes a relatively more important source. Where there is a major source of endemic *Salmonella* infection, such as animal-to-animal spread (e.g. on pig farms or calf units) or persistent environmental contamination (e.g. commercial layer farms), feed may be a relatively less prominent source of infection.

5.5.2 Assessment of the extent to which feedingstuffs contaminated with *Salmonella* can contribute to the contamination of food produced from animals

Egg contamination in the EU, typically by *S.* Enteritidis, is not likely to be greatly influenced in the short term by feedstuffs, as these are uncommonly contaminated by *S.* Enteritidis. However, in Japan, correlations between both the degree of contamination and the strains of *S.* Enteritidis present in feedstuffs and eggs have been demonstrated (Shirota *et al.*, 2000, 2001). Also, feed has been postulated as a possible initial source of epidemic *S.* Enteritidis before trade in infected breeding stock became the predominant route (Evans *et al.*, 1999). More recently, a link between pelleted broiler

feed contaminated with various *Salmonella* serotypes, including a particular phage type/genotype of *S*. Enteritidis, and raw chicken nuggets and strips has been reported from Canada (Bucher *et al*., 2007). This possible link between feed and food and subsequent human exposure of serotypes commonly pathogenic to humans can also be considered in the EU where, as can be seen from the EFSA zoonoses reports (EFSA, 2006a, 2007a), *S.* Enteritidis as well as *S.* Typhimurium occasionally were isolated from feed. Milk and other dairy products are another potential route for *Salmonella* infection of humans, and evidence for such a route from feedstuffs to dairy cow to milk has been presented (Knox *et al*., 1963).

As the link between feed contamination by *Salmonella* and infection of animals has been established, and the level of *Salmonella* contamination of animals arriving at abattoirs can affect the level of contamination of carcases leaving the plant (Campbell *et al*., 1982), then it is logical to suppose that contamination in feedstuffs can affect contamination in meats, and this has been shown in some cases. The potential route via eggs or milk is even more direct. However, the chain of transfer is unlikely to be uniform or straightforward. If the food product is effectively heat treated (for example, milk), then the long-term risk from contamination is likely to remain low for the public, if not for the farm workers. The passage of *Salmonella* through an abattoir may be considerably reduced by hygiene and decontamination processes, and there exists the possibility that a *Salmonella* strain in incoming animals is supplanted or joined by a previously introduced strain in the plant.

Food is the major route of transmission of non-typhoidal *Salmonellae* to humans (Crump *et al*., 2002; Mead *et al*., 1999), and animal food products (meat, eggs and dairy) are the vehicles primarily implicated (Anon., 2006a; EFSA, 2006a, 2007a). Eggs and chicken products are particularly strongly represented in recent data (Anon., 2005), and confirmed foodborne outbreaks of human salmonellosis in the EU show a heavy predominance of *S.* Enteritidis, with *S.* Typhimurium being the second in rank. The same report also ranks Enteritidis first amongst serotypes isolated from eggs and broiler meat, whilst Typhimurium predominates in isolates from pig meat, and is also prominent in isolates from beef and chicken. It has been demonstrated that *Salmonella* strains, including *S.* Typhimurium, from broiler feed sources can correlate with those found in birds and on derived broiler meat (Corry *et al*., 2002; Davies *et al*., 2001; MacKenzie and Bains, 1976; Pennington *et al*., 1968; Semple *et al*., 1968). In a Danish risk analysis, pork and beef were proposed as vehicles for *Salmonella* originating in pig and cattle feedstuffs, accounting for approximately 2% of human *Salmonella* cases (Hald *et al*., 2006). Another study (Newell *et al*., 1959) uncovered evidence of links between *Salmonella* contamination of pig feedstuffs, slaughter pigs and pork products.

It would appear that the risk of feedstuff-acquired *Salmonellae* appearing in human food is greatest for those livestock species where unapparent

infection is usual (i.e. chickens, pigs and turkeys) and which commonly maintain serotypes that are seen regularly in feedstuffs and are established to be of high virulence in humans, the prime example being *S.* Typhimurium.

5.5.3 Assessment of the extent to which feedingstuffs contaminated with *Salmonella* can contribute to the prevalence of *Salmonella* cases in humans

The overlap between *Salmonella* serotypes commonly found in animal feedstuffs and those isolated from human cases of salmonellosis is limited, but across the EU four of the serotypes ranked in the top 10 feed isolates (Infantis, Typhimurium, Agona and Enteritidis) are also in the top 10 public health serotypes (EFSA, 2006a, 2007a). This at least suggests the potential for feedstuff strains to pass far enough up the food chain to cause human disease. *S.* Hadar was found in Britain in poultry offal meal imported from Israel in 1969, and it became endemic in turkey breeding flocks in 1973–74 (Watson and Kirby, 1985). Within a few years it moved from a very rare to a very frequent human isolate in the UK, and a route to humans via turkey products was established (Rowe *et al.*, 1980).

There have been a number of historical reports which establish with some confidence a direct link from human salmonellosis cases through animal products to animal feedstuffs. Bone meal contaminated with *S.* Hadar and fed to chickens was linked to infections contracted from eating chicken livers (Hirsch and Sapiro-Hirsch, 1958). Meat and bone meal contaminated with *S.* Heidelberg was implicated in a milkborne outbreak of the same serotype (Knox *et al.*, 1963). In 1968, linked papers (Pennington *et al.*, 1968; Semple *et al.*, 1968) reported that an outbreak of *S.* Virchow in humans was traced back to a poultry enterprise, where there was contamination of both the hatchery and the food; a primary or secondary role for feed contamination was postulated. Possibly of most significance is the reported novel appearance of *S.* Agona in the USA, the UK, the Netherlands and Israel in 1969–70 (Clark *et al.*, 1973). Investigations in each country established a chronological sequence of isolations from Peruvian fishmeal, livestock and then humans, and a detailed study in the southern USA traced human infection back via chickens to imported Peruvian fishmeal. This case has particular impact because of the subsequent sustained level of poultry and human infections (>1 million cases) with this serotype over the subsequent two decades (Crump *et al.*, 2002). A similar situation is currently occurring with *S.* Agona, which regularly contaminates vegetable proteins and is found in infections in turkeys. *S.* Rissen is another *Salmonella* serotype which appears to have passed from vegetable proteins to turkeys in recent years, but is still uncommon in humans (Anon., 2006b). In a Danish study (Hald *et al.*, 2006) it was estimated that up to 2.1% of the domestically acquired human salmonellosis cases in the period 1999–2003 could be attributed to feed-borne serotypes.

Despite such evidence, differences in serotypes isolated from humans and from feedingstuffs are sometimes used as an argument to claim that feed does not contribute substantially to human food-borne illness (Crump *et al*., 2002). Several aspects have to be considered, such as the efficiency of sampling of feed-producing facilities, and the 'filtering' effect which relates to the infectivity and pathogenicity of different serotypes in different hosts. It is also likely that feed may have been involved in the international dissemination of 'epidemic' strains of *Salmonella* such as *S.* Typhimurium DT104 (Davies, 2001; Helms *et al*., 2005) and *S*. Enteritidis (Evans *et al*., 1999).

5.5.4 Considerations on the risk posed by feed as a contribution to *Salmonella* infections in animals and humans in comparison to other possible sources

Although opinions vary on the importance of feedstuffs contamination (Jones *et al*., 2004) there is a substantial body of evidence that in many situations feedstuffs can pose a significant risk of *Salmonella* infection for humans and animals. Although there is limited overlap between common human and feed serotypes, the aggregation and ranking of data on serotypes may obscure regional patterns. For example, serotypes Enteritidis and Typhimurium appear to be uncommon in feed in many reports (Bisping, 1993) but more common elsewhere (MacKenzie and Bains, 1976). Some authors consider feed to rank alongside imported pigs for the risk of *Salmonella* introduction (Sauli *et al*., 2005), and the link between feedmills and *Salmonella* in pigs is well established (above), whilst on-farm mixing of feed may also be a risk factor for introduction of *Salmonella* in pig herds despite being partially protective in terms of the risk of a high within-herd prevalence of endemic *Salmonella* (Davies *et al*., 2004). Feed is cited by some authors as a major source for cattle herd infections (Eddy, 2004; Jones *et al*., 2004).

In broiler production, both hatchery and feed contamination are implicated in *Salmonella* strains seen at slaughter (Corry *et al*., 2002), and among UK layer flocks the use of certain feed mills is a risk factor for *Salmonella*, including *S.* Enteritidis (Snow *et al*., 2007). This last point is unexpected, as *S.* Enteritidis is currently uncommonly found in UK feedstuffs. Feed contamination, including the use of poultry offal and feather meal as well as indirect contamination of other feed materials from environmental sources, is likely to have played a greater role in the early stages of the *S*. Enteritidis epidemic in UK poultry. However, feed sampling may only reveal part of the picture, as sample size has a substantial effect on *Salmonella* recovery (Shirota *et al*., 2000), and there is also the filtering effect of farm and animal environment to consider, potentially resulting in certain occasional contaminants being ultimately more successful colonizers than some of the more common isolates.

The effects of feed contamination should also be considered on short-term and long-term timescales. With short-cycle production such as broilers, feed contamination can be seen to introduce new serotypes and to fairly quickly influence the serotypes seen at slaughter and on carcases (MacKenzie and Bains, 1976). The effects on longer-term production cycles that may have endemic *Salmonella* strains, such as in layers and pigs, may be more subtle but ultimately still significant, particularly when efforts to reduce the level of infection are being made in a unit or if the feed-related *Salmonella* gains access to the higher levels of pig breeding organizations and integrations.

In conclusion, in the same way as *Salmonella*-contaminated food is the major source of *Salmonella* infections in humans, animals face a similar risk of becoming infected when fed with *Salmonella*-contaminated feed. Animals such as poultry and pigs which derive all or the greatest part of their nutritional requirements from compound feed are most at risk, but the relative importance of feed as a source depends on the coexistence of other sources of infection. Such predominant sources include movements of infected animals, infected wildlife vectors or residual environmental contamination, for which the primary source may have been *Salmonella*-contaminated feed. The importance of *Salmonella* infection from feed varies according to the position in the breeding and production pyramid where infection occurs. For example, if infection occurs in a primary pig or poultry company, it may be distributed worldwide by international trade in breeding stock, as well as resulting in perpetual infection on continuously occupied primary and commercial breeding units, which then becomes an ongoing source of *Salmonella* for an indefinite period. Occasional introductions of *Salmonella* into parent breeding or commercial broiler and turkey flocks or fattening pig herds which are operated on an all-in/all-out basis present a shorter-term risk, but may persist if cleaning and disinfection is not effective, or may contaminate hatchery or abattoir equipment, which may occasionally also be a long-term problem.

5.6 Considerations on the possible establishment of microbiological criteria for *Salmonella* in feed

5.6.1 Background: microbiological criteria as defined in the EU legislation

EC Regulation 2160/2003 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:325:0001:0015:EN:PDF) on the control of *Salmonella* and other specified food-borne zoonotic agents aims to ensure that proper and effective measures are taken to detect and control *Salmonella* and other zoonotic agents at all relevant stages of production, processing and distribution, including in feed, in order to reduce their prevalence and the risk they pose to public health. Those specific requirements should be based

on targets for the reduction of the prevalence of these agents in animal populations, mainly at the level of primary production and, where appropriate at other stages of the food chain, including in food and feed, and in accordance with Article 5(3) of Regulation (EC) No. 183/2005 on feed hygiene, specific microbiological criteria and targets shall be adopted. An EU Food Safety Criterion defines the acceptability of food products. If the criteria are not met the product/batch must not enter the market, and it has to be withdrawn if it has been placed on the market. An EU Process Hygiene Criterion gives guidance on, and is an indicator of, the acceptable functioning of HACCP-based manufacturing, handling and distribution processes. It sets indicative contamination values above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law.

Due to the statistical limit of sampling plans, microbiological testing of food and feed for pathogens occurring at a low prevalence may give a false feeling of safety if a sufficient number of samples are not tested over time. Therefore, for pathogens present at a low frequency, the risk of not detecting contaminated lots is high (ICMSF, 1986). In these cases efficiency of applying food safety criteria on food/feed to improve animal and consumer protection will be low.

5.6.2 Microbiological criteria for *Salmonella* in feed

Because of the possibility of animal infections, the objective should be that *Salmonella* should not be present in animal feed. Different surveys have found a low prevalence of *Salmonella* in compounded feeds. However, the limitations of the available methods for detecting a low prevalence of contamination make the efficacy of establishment of a feed safety criterion based only on the end product questionable, as this would also require storage of the feed during the testing procedure before delivery. A more efficient option could be the establishment of one or more process hygiene criteria at certain critical stages in the production chain, including in the end product.

5.6.3 Microbial testing and establishment of process hygiene criteria as an integrated part of individual HACCP-based programmes

According to the feed hygiene directive 183/2005 (http://europa.eu.int/eur-lex/lex/LexUriServ/site/en/oj/2005/l_035/l_03520050208en00010022.pdf) feed business operators shall put in place, implement and maintain a permanent written procedure or procedures based on the HACCP principles. In addition they must implement effective monitoring procedures and establish corrective actions when monitoring indicates that a critical control point is not under control.

Testing for *Salmonella* along specified places in the production line and in the end product should be an integrated part of an efficient HACCP

programme, and the isolation of *Salmonella* should be notified. Ideally, preventive action should start at the level of primary production. The importance of starting the control already at the crushing and the rendering plants is emphasized, in contrast to the currently often applied practice of focusing only on the feed mills. For animal-derived products, prevention to a varying extent is already in place during the primary (animal) production, but has to be focused also on the rendering plants. Control at the crushing and rendering plants should, when applicable, follow the steps that are described below for the feed mills.

Testing of incoming raw material
Feed materials could be classified according to their risk of being contaminated by *Salmonella* and those identified as a risk should be monitored before intake. The monitoring of batches of feed material has the same limitation for the probability of detection of *Salmonella* as for the monitoring of compounded feed described earlier. Sampling according to Ekbohm (1993) could be applied, which takes into consideration an uneven distribution of *Salmonella* contamination.

The actions to be taken if lots of ingredients are found to be positive for *Salmonella* will depend on the specific process that follows, and the specific HACCP programme, but may include rejection, decontamination, contact with the producer, etc.

Testing at key sampling points along the compound feed production chain
The following places along the production chain are normally regarded as key sampling points in feed mills for production of compound feed and when applicable also in crushing and rendering plants:

- Unloading pit for feed materials
- Ingredient sieve or ingredient bins
- Filter aspirating the production line
- Pellet or meal cooler
- Pellet shakers and crumblers
- Finished product bins or outloading gantries.

Samples of dust from these places should be tested for *Salmonella* at regular intervals. If samples are tested positive, corrective actions should be taken, which could include cleaning and disinfection, increased monitoring, stopping production and stopping delivery.

A common EU process hygiene criterion for *Salmonella* in the production chain, at one or more of the above key sampling points, could be considered.

Testing of compound feed
Testing of compound feed for *Salmonella* should be used as part of an integrated method to validate the efficiency of the HACCP-based control

programme. An overall requirement should be that the final feed is free from *Salmonella*. The samples should be taken in a way that maximizes their representativeness of the batch. If samples are tested positive then corrective actions should be taken, including (1) investigation of the critical control points, (2) investigation of raw material records, (3) the effective treatment regime, and (4) increased intensity of sampling and testing of production.

It is suggested that a common EU process hygiene criterion for compound feedingstuffs is established according to what is described above.

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6

Detection and identification of animal by-products in animal feed for the control of transmissible spongiform encephalopathies

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Abstract: This chapter discusses the official light microscopic method for the detection and identification of animal by-products in feed. The legal framework in force is summarised, with the focus on future legal changes in the actual feed ban. A comparison of the official method with alternative ones (PCR, immunoassays and NIR techniques) concentrates on the respective advantages and disadvantages of the different approaches. Future scientific issues are highlighted, as is the need for an integrated approach combining the various existing methods to take advantage of their complementary features.

Key words: animal by-products, legislation, microscopy, PCR, near-infrared microscopy, immunoassays.

6.1 Introduction

The outbreak of the transmissible spongiform encephalopathies (TSE), and more particularly of bovine spongiform encephalopathy (BSE) in the United Kingdom in 1986, underlined the need for the control of animal feed in order to stop the spread of these diseases. As recommended by the World Health Organization (WHO) and other international bodies and agencies, measures for public health protection include the prohibition of intra-species recycling (sometimes incorrectly referred to as cannibalism) in animal feed. Many countries therefore imposed severe regulations banning the use of animal by-products and, in particular, meat and bone meal. In the European Union (EU), Commission Regulation EC/1234/2003

confirmed the prolongation of the existing total feed ban because of the lack of reliable species-specific methods for identifying animal proteins. When such methods do become available, the present ban will change to a species-to-species ban in line with provisions from Commission Regulation EC/1774/2002. This chapter discusses current methods for animal protein detection in feed in the context of the current legal framework and its future development. The focus is on the performance and shortcomings of the official microscopic method compared with alternatives such as polymerase chain reaction (PCR), immunoassays and near-infrared (NIR)-based methods. Marking prohibited animal by-products with chemical tracers is also discussed, as it constitutes not only a way to follow up the categorisation of animal by-products, but also an interesting pre-detection technique for exposing fraud.

6.2 Legislative framework and requirements

The EU's current legal framework relating to the use of animal by-products is very complex because it results from modifications and amendments of successive legal texts. The historical background to the framework is presented by van Raamsdonk *et al.* (2007) and Fumière *et al.* (2009).

The legislation currently in force can be summarised as follows. Commission Regulation EC/1069/2009 groups animal by-products into three categories in terms of their TSE risk. *Category 1*, for example, includes specified risk material that, along with all other animal by-products, has to be incinerated, buried or properly treated before final disposal. *Category 2* includes animal material such as manure, intestinal content and other by-products that can be used for soil amendment by composting or biogas production, but are excluded for animal feed purposes. *Category 3* includes animal products or carcasses initially regarded as suitable for human consumption. This category of material can be used for feed production under strict conditions and processing methods given in the annexes of Regulation EC/2002/1774 (e.g., method 1 is mandatory for mammalian material). The regulation also defines the concept of processed animal proteins (PAPs) that was amended further in Commission Regulations EC/808/2003 and EC/829/2007:

> 'Processed animal protein' means animal protein derived entirely from Category 3 material, which have been treated in accordance with [the provisions of the animal by-product regulation and its amendments] so as to render them suitable for direct use as feed material or for any other use in feedingstuffs, including petfood, or for use in organic fertilisers or soil improvers; however, it does not include blood products, milk, milk-based products, colostrum, gelatine, hydrolysed proteins and dicalcium phosphate, eggs and egg-products, tricalcium phosphate and collagen.

So in fact, said in another way, PAPs do correspond to meat and bone meal made out of low-risk material as well as fishmeal and integrates also their correct processing.

Commission Regulation EC/1432/2007 lays down the control principles on marking and transport tracking for each category. It requires the use of permanent marking by smell and glyceroltriheptanoate (GTH) for Category 1 and 2 materials in processing plants in order to ensure complete traceability. Animal products in Category 3, depending on their taxonomic origins, are subject to the restrictions in use in order to avoid intra-species recycling, which is the primary intention of Commission Regulation EC/1069/2009. It should be noted that the legislation does not provide a clear definition of what is meant by the term 'species'. It refers only to ruminants (bovines, ovines and caprines), porcines, poultry and fish; these are not species but superior taxa. This lack of precision could lead to confusion and misinterpretation of the current legislation with regard to animal groups. A schematic summary (Fig. 6.1) of the current situation with respect to the use of PAPs obtained from Category 3 material is provided here (by courtesy of A. Boix, adapted).

Feeding ruminants with PAPs originating from mammals is a permanent prohibition stipulated by Commission Regulation EC/999/2001. Some categories of mammalian products, however, are authorised (including milk and dairy products, gelatine, hydrolysed proteins below 10 kD produced under given conditions, blood derivatives that do not originate from ruminants, and dicalcium and tricalcium phosphates). As there are

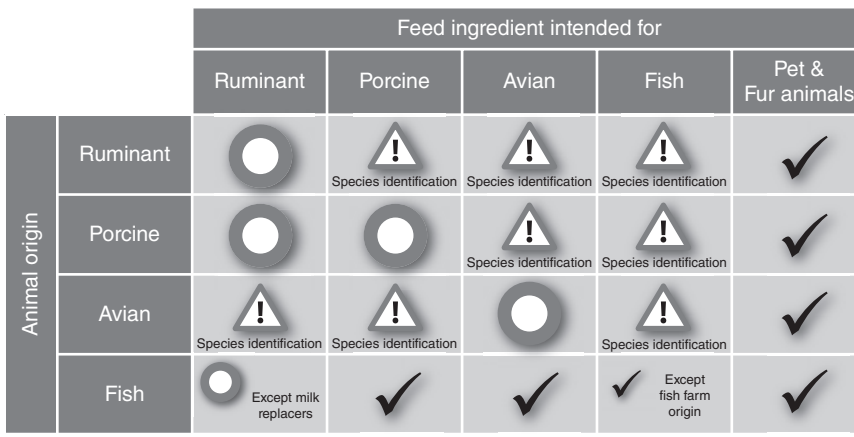

Fig. 6.1 Overview of the current feed ban in Europe. Circle signs refer to permanent prohibition; triangular warning signs refer to future possibilities of lifting the prohibition provided species identification is possible. Tick marks refer to authorised use.

no methods yet for all species-specific identifications of PAPs, the species-to-species feed ban proposed by Commission Regulation EC/1774/2002 cannot be implemented as it is, and it has therefore been amended by Commission Regulation EC/1234/2003, which confirms the extended feed ban and will be reviewed only when new scientific evidence and new methods emerge. The use of PAPs of mammalian and avian origin remains prohibited in animal feed. The use of fish meal is generally authorised in the EU as feed or an ingredient of animal feed with a few restrictions. Commission Regulation EC/1234/2003 stipulates that fish meal and fish by-products originating from farmed fish are prohibited in feed for fish of the same species, but fish meal originating from fish caught in the open sea is authorised. The use of fish meal for ruminants is restricted to milk replacers for young ruminants, as stipulated recently in Commission Regulation EC/956/2008. There is almost no restriction on the use of PAPs for pets and fur animals which are, by definition, not intended for human consumption.

There is no gradation in the application of the feed ban in the EU. In other words, zero tolerance is applied as soon as prohibited animal traces are found in a sample (European Commission, 2005; van Raamsdonk *et al.*, 2007), which is then declared to be positive. The only exception to this, as stipulated in Commission Regulation EC/163/2009, concerns the feeding of farm animals with ingredients of plant origin in which there are insignificant amounts of bone fragments. This is authorised provided there has been a favourable risk assessment on the possible source of the contamination and the final destination of the ingredient consignment. A typical historical example for this is the naturally occurring presence of bone spicules in sugar beet pulp.

6.3 Future legislative trends in the EU

According to the Commission's TSE Roadmap (European Commission, 2005), some modifications could be made to the BSE measures while continuing to give high priority to food safety and consumer protection. Different options are presented when certain conditions are met. They include the introduction of tolerance levels that could for instance be applied to a limited presence of fish meal in ruminant feed. This presence might originate from cross-contamination by feed for non-ruminants in which fish meal is authorised. This option would reflect a more risk-based policy, but it does depend on the development of a more robust quantification method that is not currently available, as demonstrated in Veys and Baeten (2007a). In order to lift the total feed ban for non-ruminants, and implement a species-specific feed ban, the use of non-ruminant proteins in feedstuffs, excluding intra-species recycling, could be an option if methods for species identification are available. Fumière *et al.* (2009) noted that such tests are still under

development and therefore not yet validated, which means that any modification of the feed ban in EU is still pending.

6.4 Detection and identification of processed animal proteins

6.4.1 Official method

Annex VI of Commission Regulation EC/152/2009 describes the official analytical method in the EU for the detection of PAPs in animal feed. This regulation authorises detection only by light microscopy for official controls, but does not exclude alternative methods for confirmatory purposes provided the first analyses are carried out by light microscopy. Commission Regulation EC/152/2009 is a revamped version of the repealed Commission Directive EC/126/2003, based on past studies intended to harmonise and improve the detection method throughout EU Member States. Those studies revealed large variations in the specificity, sensitivity and accuracy of the initial method. Gizzi *et al.* (2003, 2004), von Holst *et al.* (2004) and van Raamsdonk *et al.* (2005) provide the historical background to this.

The official microscopic method deals with two aspects of the presence of PAPs in feed: the detection *in se* (qualitative analysis); and the estimation of the amount of the contaminant (quantitative analysis). Both aspects are discussed here.

Qualitative determination by light microscopy is conducted on the fractions obtained from the feed or ingredient material, after grinding if necessary: the sieve fractions of the raw material, and the concentrated fraction (or sediment). Both fractions have to be prepared from a minimal representative portion of 5 g of the ground material. The sediment is obtained after settling the raw material in tetrachloroethylene (TCE). This settling process concentrates all particles with a density above 1.62, such as bones, fish scales, teeth fragments and cartilage particles, as well as minerals. A certain degree of freedom is allowed regarding the laboratory equipment used for sedimentation; either conical-bottomed settling beakers or separation funnels can be used. The different fractions also need to be put through a 500 μm square mesh sieve in order to separate the large particles from the smallest ones, prior to visual analysis with the appropriate optical equipment. Both stereomicroscopic and transmitted light microscopic analyses are therefore required. The use of the microscope is restricted to the fine sieve fractions. From these fine sieved fractions, microscopic slides are prepared and mounted. Various mounting media, including glycerol or paraffin oils, are allowed for slide preparation, provided that their physicochemical properties allow the air inside the bone lacunae to be maintained as long as possible, making them more easily detectable. The official protocol also authorises the use of different staining reagents for enhancing structures such as bones, fish bones, fish scales, hairs and feathers that can be coloured

by Alizarin Red and cystine reagens, depending on their respective chemical composition.

Many collaborative studies (Veys and Baeten, 2007a, 2007b, 2008; van Raamsdonk *et al.*, 2008) have focused on the accurate application of the microscopic method and its global reliability in control laboratories. In 2006 an interlaboratory study (Veys and Baeten, 2007a) involving the EU's network of National Reference Laboratories (NRL), organised by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP) reached a series of conclusions on the qualitative detection performance of the microscopic method. The percentage of participants giving a faultless answer set was 55%. Some 77% of the participants obtained a consolidated accuracy (i.e., the ability to correctly detect the presence or absence of terrestrial MBM and fish meal in feed) above 0.95. This indicated that the method was suitable for detection purposes. The lowest observed sensitivity value was 0.88 (from 66 analyses), obtained from a material adulterated with 0.1% terrestrial MBM and 5% fish meal. This was the best performance obtained for this type of material in the EU since studies following this protocol began (Veys and Baeten, 2007a). Despite the good performance globally, improvements are still needed, especially with regard to sensitivity in the detection of terrestrial particles when both terrestrial MBM and fish meal are present in a feed. Actually, false negatives in this type of feed occur more frequently (van Raamsdonk and van der Voet, 2003; Gizzi *et al.*, 2004; von Holst *et al.*, 2006) because the fish particles tend to mask the terrestrial ones.

In 2007, another proficiency test organised by the EURL-AP and involving the same network of participants gave a proportion of 68% faultless answer set, while a sensitivity value of 0.84 (from 25 analyses) was found for 0.1% terrestrial MBM- and 5% fish meal-adulterated material (Veys and Baeten, 2007b). The overall improved performances within this network of laboratories indicate that detection skills can be improved by continuous training and regular proficiency evaluation.

The proficiency test for the detection of animal proteins, conducted on behalf of IAG and organised by RIKILT in 2008, also delivered good results on the microscopic method (van Raamsdonk *et al.*, 2008). Of those participants who followed the Commission Directive EC/126/2003 recommendations in conducting this ring test, 70% scored faultless. In the same study, the sensitivity in terrestrial particles by detection classical microscopy was particularly high for material adulterated by only 0.05% terrestrial MBM with a value of 0.95 (from 43 analyses). This demonstrates that still very satisfying performances can be reached at levels of contamination with terrestrial MBM half below the commonly described limit of detection (LOD) of 0.1%.

In 2008, a proficiency test organised by the EURL-AP (Veys *et al.*, 2009) showed that 62% of the participants obtained excellent results (a consolidated accuracy above 0.90 calculated on 10 blind samples). This study also produced high scores even for samples adulterated with 1% pure muscle

MBM containing almost no bones (sensitivity of 0.86 for the detection of animal particles). Intriguingly, in the same collaborative studies referred to in this chapter, even in the most recent studies, a relatively high percentage of false positive results for the presence of fish was observed. A possible reason for this was given by van Raamsdonk *et al.* (2008). They noted that some bone particles from the terrestrial meal batch used for the sample preparation were not easily recognisable as bone, possibly because of the heating treatment and therefore not independent of temperature, as thought till recently (Gizzi *et al.*, 2003; Sanches *et al.*, 2006; van Raamsdonk *et al.*, 2007). It can therefore be presumed that at least some of these particles had been misinterpreted and possibly characterised as fish, although there was no direct evidence for this. Additional information, however, might more accurately explain this rather low specificity for fish. Some terrestrial bone particles, such as fragments originating near the central osteon canals along the diaphyse of long bones (Di Fiore, 1967), behave like fish bone, with remarkably typical very elongated lacunae and a radiating network of canaliculae (Veys, unpublished communication at IAG annual meeting Budapest 2008). This might have led to some logical but unexpected misidentifications. Veys *et al.* (2009) confirmed this hypothesis by showing pictures of pure bovine bone fragments presenting lacunae with a surrounding network of canaliculi similar to those observed in fish bone fragments. In addition some other microscopic fragments could also be sources of misinterpretation: plant trichomes can be misconstrued as fish teeth, and some plant particles could be mistaken for fish bone fragments or even otoliths. Although those observations might appear to be anecdotal because collected data on the performance of the method indicated its reliability, what is nevertheless underlined is that the faultless visual detection of prohibited ingredients in feed depends greatly on the microscopist's experience and skills. Moreover the studies also show that continuous research on new markers – both microscopic features of animal particles and new staining methods – is absolutely necessary.

Even if the official method for qualitative analysis offers sufficient reliability as it stands, at least for frontline purposes, results from past studies also stressed the need for further fine tuning of the method. Actually too many aspects of the method are subject to interpretation because of a lack of precision. Claims that deviations between qualitative results are linked to these aspects are regularly made (e.g., Veys and Baeten, 2007a; van Raamsdonk *et al.*, 2008). For instance, the initial portion of at least 5 g of sample material to be used for preparing the different fractions should be fixed at a higher value. Although from past tests there is no statistically significant evidence of any correlation between the amount of the initial portion used for the sedimentation and sensitivity scores, it is suggested that a fixed value of 10 g should be used in the future. Apart from statistical theoretical models and reflections (Murray *et al.*, 2005; van Raamsdonk *et al.*, 2008), the reason for this amount is clear: the more material used, the

more sediment can be recovered. This is crucial, especially when contamination levels below 0.1% PAPs have to be detected, indicating scarce animal particles in the feed: more sediment leads to a higher probability of presence of animal particles. The diversity of vials for recovering sediment could also be a source of heterogeneity (van Raamsdonk and van der Voet, 2003), at least with regard to the reproducibility of qualitative analysis because of quality variations in the obtained sediment, such as the relative concentrations of minerals vs. bone content. This could also partly be a source of variation in quantitative analyses because the type of vial could account for variations in recovered sediment weights (van Raamsdonk *et al.*, 2008). Gizzi *et al.* (2003) stressed the need for harmonised slide preparation for obtaining comparable results among laboratories, but this remains unchanged. On the use of mounting media, the official protocol does not specifically refer to any measurable viscosity unit that would be required for glycerol and for the most variable chemicals such as commercial paraffin oils. Embedding media that are too fluid, for instance, should be avoided because due to rapid filling of lacunae, the number of bone particles could be overlooked. Recently the use of Norland Optical Adhesive 65 for permanent slide preparation has been proposed by Veys and Baeten (2010). This resin preserves all the optical properties needed for distinguishing bone fragments, while offering long-term conservation of slides. The number of slides that need to be observed is also discussed because of unclear instructions in the different cases. Instructions on the type of slide – hollow or classical, size of coverglass, amount of material to spread on a slide, density of the slide (number of particles over unit area) – are also lacking. Fixing similar parameters for the use of the microscopic method is needed because it is still dependent on versatile parameters – the analyst's skills and human observation capabilities – which are both subjective.

Clear decisions on all of the above issues will provide the basis for a more reliable version of the official Commission Regulation EC/152/2009 protocol upon which more uniform, if not more reproducible, qualitative decisions among different laboratories will be made. This is particularly important when two accredited laboratories work on the same sample material or conduct counter analysis. The current – and not questionable – zero tolerance policy regulating the feed ban will be effective only if a method strictly applied by two operators to a same material is able to reproduce same results expressed in the same way (or at least with a low rate of discrepancy in results). This might not be the case with the current method; some amendments are also needed with regard to the expression of results. Effectively in cases of very low contamination levels (e.g., <0.01%), one could find only a single particle of animal origin. According to legislation, the sample should then be declared as positive, but statistically this is nonsense because such results are not repeatable and therefore the risk of false positive results is increased. There is still not even a defined limit of detection (LOD) for the detection of PAPs in feed by microscopy.

Various values have been mentioned in the literature, ranging from 0.1% in Commission Regulation EC/152/2009 to 0.05% (Sanches *et al.*, 2006), but these values are debatable because there is no method for defining LODs for qualitative methods delivering binary results (present or absent). For natural cross-contaminants (e.g., from rodents or birds) only a few animal particles are likely to be found. Extra information straight from the scientific observations could therefore be needed. Information such as the number and type of particles being detected (e.g., hairs, teeth, claw fragments) should be mentioned in order to determine the possible origin of the contamination. This would also force laboratories to pay more attention to still occurring and never totally avoidable accidental laboratory cross-contamination, or at least not exclude this possibility when very few particles are detected.

Commission Regulation EC/152/2009 also includes a quantitative method for estimating PAPs in feed. Legally, quantification is currently voluntary, not mandatory. Where official analyses need to refer to an estimation of the amount of animal constituents, EU Member States are asked to use this method. The quantification can be carried out only on the sediment provided it contains bone particles or other animal identifiable fragments. The calculation is computed by using the formula

$$\frac{S \times c}{W \times f} \times 100$$

where S is the sediment weight, c (or d in case of fish) is a correction factor for the estimation of the portion of terrestrial bones (or fish bones and scale fragments) in the sediment, W is the weight of the sample material used for the sedimentation and f is a correction factor for the proportion of bones – including fish bones and scales – in constituents of animal origin in the sample examined, depending on the type of PAPs present.

The non-compulsory character of the method, as well as the total feed ban policy and the exclusion of any tolerance or threshold value, partly explains the absence of any extended study on the quantification until recently. From the few data available, those from the STRATFEED project indicated that calculations based on this semi-quantitative method are unreliable or scientifically impossible (van Raamsdonk *et al.*, 2005). This verdict relied on the fact that f can never securely be estimated; this was also reported by von Holst *et al.* (2006), who considered quantification to be almost impossible because of a lack of information on the type of PAPs being detected in a blind sample. As specified earlier, the relaxation of some measures of the present ban depends on the introduction of tolerance levels based on a reliable quantification method. That is why the CRL-AP ILS 2006 study investigated the implementation and evaluated the performance of this quantification method based on light microscopy (Veys and Baeten, 2007a). Based on the quantification of sets of five blind fish-adulterated feed

samples, the study showed that a third of the participants were unable to apply the method. The results from the remaining two-thirds showed a global overestimation and poor reproducibility (RSD_R ranging from 85 to 116%). The repeatability was nevertheless satisfying (RSD_r ranging from 12 to 30%). Veys and Baeten (2007a) concluded that the main source of variation was probably the *d* factor and not the sedimentation process (or *S* and *W* parameters) or the *f* factor. Although the latter affected only the observed overestimation, it was suggested keeping it as a constant since it cannot be calculated from a blind sample. Two arguments support the hypothesis that *d* might be the main source of variation. The first one relates simply to the absence of any guidance for estimating the *c* and *d* factors in the EC 152/2009 regulation. The second one is that this factor depends entirely on the ability of the microscopist to discriminate between bones (terrestrial or fish) and scales and particles of another nature from the sediment.

Based on the CRL-AP ILS 2006 study, a new protocol aimed at setting the conditions for estimating *c* and *d* has been developed and published (Veys and Baeten, 2010). This protocol is based on a stereological approach using grid counting for estimating the proportion of terrestrial bones, fish bones and scales in the sediment. The in-house validation of this protocol showed a major improvement in intra-laboratory reproducibility (Veys and Baeten, 2010). A larger-scale study (Veys and Baeten, 2008) using a similar protocol (i.e., based on grid counting for evaluating *d* in a standard way) was organised in 2007 with the same participants as for the CRL-AP ILS 2006 study. This time, all the participants were able to apply the protocol, which had to be followed strictly in all its aspects (i.e., sediment staining, grid counting, same number of slides and number of fields to observe, fixed value for *f*). In spite of these standardisations, the results were still not satisfactory in terms of further validation. The inter-laboratory reproducibility was still poor, albeit slightly improved. Statistical analyses of the results not only demonstrated that the combined impact of the sedimentation process and staining on the quantification variability was minor, but also proved that there was a direct major impact of *d* on the final estimation of PAP content. The authors concluded that the parameters that could affect the determination of *d* are slide heterogeneity, number of slides and fields to observe, potential misinterpretation of some stained particles described as bones (see the discussion above on qualitative analysis), and the skills of the analyst. On the issue of an analyst's ability to discriminate animal particles from other particles, Veys and Baeten (2008) consider that this could be achieved only by scientific experience and the correct use of the microscope, as it is known that it can lead to erroneous estimations. Thus, it is reasonable to assume that light microscopy will not remove the subjective aspect of quantification. The quantitative estimation method described in Commission Regulation EC/152/2009 has proved to be deficient. Attempts to improve it have not yet led to a fit-for-purpose protocol, but progress and research are ongoing.

Data collected from the past studies therefore show the suitability of light microscopy-based detection of PAPs in feed, but demonstrate its weakness in the quantitative estimation of PAPs content. Although the qualitative results are of high quality, the correct identification of particles (animal vs. other types) appears to be the keystone of the quantification method. Other identification methods that are less subject to human interpretation could be very valuable alternatives or complementary approaches for the detection of PAPs in feed, especially when an ingredient of animal origin lacks microscopic features. It should also be noted that the microscopic detection of PAPs and the characterisation of their origin is limited to terrestrial and fish groups. Characterisation at lower taxonomic levels is very difficult, if not almost impossible, in terms of direct observation using light microscopy. Other methods are therefore needed for the determination of these species.

6.4.2 Alternative methods

Currently available optimisation methods for detecting PAPs in feed and determining their origin rely on PCR, immunoassays and NIR-based methods. An exhaustive review of the literature on alternative methods was compiled by Fumière *et al.* (2009). This chapter presents an outline of these methods, pointing out their advantages and disadvantages.

Compared with light microscopy, use of near-infrared microscopy (NIRM) is the most similar approach because it is a particle-based analysis. Basically the principle of NIRM is derived from near-infrared spectrometry (NIRS) combined with a microscope. The identification relies on the spectral absorbance analysis of single particles after exposure to a near-infrared beam. The application of NIRM for detecting animal proteins in feed has long been pioneered by the Walloon Agricultural Research Centre (CRA-W) since the publications by Piraux and Dardenne (1999, 2000) on the potential of NIRM for feed identification. Major improvements were achieved through the European STRATFEED project (Baeten *et al.*, 2004). The method involves spreading particles from raw feed on a sample holder inserted under the infrared beam of a microscope (λ ranging from 1100 to 2500 nm). Each particle delivers a NIR spectrum as a signature of its molecular composition (i.e., a unique spectral identification corresponding to the nature of the particle). Libraries of spectral signatures from a wide range of feed ingredients therefore have first to be built. These libraries need to contain spectra from plant and mineral feed ingredients, as well as animal by-products (e.g., fish meal, poultry meal, mammalian, hatchery by-products, milk derivates) that are known to occur in compound feeds. Comparison by chemometric analysis of a single particle spectrum with spectra from the library will enable its identification. The advantage of the NIRM method is that it is free of the interpretation of an observer. The identification is accurate and no longer biased by a lack of expertise. Although the method

can distinguish between different plant species, as well as between particles of plant and animal origin, it cannot distinguish between different animal species. NIRM can identify fish, mammals and poultry particles, but it cannot discriminate at species level. In addition, there is an overlapping of NIRM spectra between identifiable groups, so results can be considered as informative but not as conclusive.

A great advantage of the NIRM method is that it is non-destructive; particles can be recovered after analysis for further characterisation by other methods. Furthermore NIRM has also been shown to be efficient on the sediment fraction obtained after TCE settling, as used in light microscopic sample preparation (Baeten *et al.*, 2004). NIRM has an LOD of <0.1%. As NIRM can easily estimate the numbers of animal spectra vs. spectra of other origins, there is the potential for its use in quantification, as reported by several authors (for review see Fumière *et al.*, 2009). At present, however, quantification methods based on NIRM need further investigation prior to any validation. The disadvantages of NIRM include the task of obtaining a large enough number of spectra for delivering information on the feed; this is prohibitively time-consuming, although it can be automated by mapping design. Finally the initial investment in the equipment required can also present a problem, although prices have fallen in recent years.

NIRS has long been studied for the authentication and control of food and feed in the industry. As in the case of NIRM, the principle of NIR is the spectral analysis of absorbance of near-infrared wavelengths of analytes. The characteristics of the absorbance will determine a spectral profile depending on the composition of the analyte, and more precisely, on the type of chemical bonds of major molecular groups. The technique, as commented by van Raamsdonk *et al.* (2007), has the advantage of using a larger representative portion of the sample compared with NIRM. Thus, NIRS addresses the issue of sample heterogeneity in feed, but it is considered as suitable only for screening or first-line analyses (Murray *et al.*, 2005). It is rapid, non-destructive and cost-effective, but has an LOD which is not low enough (>1% contaminations). It is therefore suitable for the gross contamination of feed with animal by-products. In addition, NIRS allows a distinction to be made only between high-level taxa (terrestrial animals vs. fish). A major disadvantage of NIRS is that it is an indirect method and therefore requires large collections of spectra for reference and good mathematical models for equipment calibration before an accurate measurement and interpretation of submitted samples can be made.

Through genetic amplification, the PCR method enables well-defined DNA sequences to be detected. This method involves the following steps. First, an extraction is done to isolate the DNA fragments that might be present in the feed. The PCR *in se* is performed on a fraction of the DNA extracted in order to produce amplicons (copies of well-defined targets). During this process, in each heating cycle the number of amplicons is

theoretically doubled. If real-time PCR is conducted, the amplicons react to a fluorescent probe generating a signal that can be followed and indicates the evolution of the number of copies of amplicons over time; this is why real-time PCR is sometimes confusingly referred to as 'quantitative PCR'. The most significant advantage of PCR is that it allows the detection of well-identified taxonomic levels; the DNA targets selected for the genetic amplification are sequences of nucleotides that are often species-specific, or specific for taxa such as a family or an order. Once a DNA target has been selected, in the PCR process only the corresponding amplicons will be multiplied, giving almost perfect specificity. Fumière *et al.* (2009) reported the existence of target sequences for mammals, birds, fish, ruminants, bovines, ovines, pigs, chicken and poultry, rats and mice.

A recent inter-laboratory study conducted by the IRMM (Prado *et al.*, 2007) on three real-time PCR methods targeting either bovine or ruminant sequences revealed that all the methods were able to detect bovine MBM at a concentration of 0.1% in feed, demonstrating the maturity of these methods. The PCR protocols for detecting and identifying animal by-products in feed, however, will always suffer from some deficiencies. The first concerns identification, as PCR is an indirect method of identification: it is the DNA in the animal product that is detected and not the type of animal product that is often composed mainly of proteins. Thus, a positive signal for bovine does not ultimately mean presence of MBM or other prohibited PAPs; it could simply originate from authorised ingredients in feed such as dairy products that contain bovine DNA. This is a real limitation in the use of PCR, which needs complementary methods, such as light microscopy, to accurately determine the nature of the animal ingredients added to feed. A second concern is the detection of DNA. European processing treatments of PAPs on mammalian material (method I) are known to be rather harsh and lead to an important degradation of the DNA molecules, mainly by a high fragmentation. If target sequences are too long (>100 bp), there is a risk that process-related degradation will mean that only shorter nucleotide sequences can be extracted, masking the presence of PAPs and leading to false negative results. Shorter sequences are therefore recommended (Fumière *et al.*, 2006). In addition, the number of copies of the DNA sequence has to be high enough or the signal amplification will be too weak or will be beyond the cut-off values. For this reason, selecting multi-copy DNA targets, as mitochondrial sequences, is recommended in order to prevent false negative results.

The possibility of using PCR for quantitative purposes is still not a reality. Quantitative results need to be expressed in terms of mass fractions of ingredients. PCR allows the quantification of the copies of a DNA target, which is influenced by the amount of DNA recovered after extraction and the number of available intact copies of this target left after the heating process. If quantification could be achieved by PCR, it should be expressed in terms of the number of amplicons. As there is no correlation between

this number of DNA copies and any effective amount of animal material expressed in mass fraction, there is no possibility of using PCR for estimating the amount of PAPs present in a feed (the same amount of material will therefore contain fewer targets if the heat treatment is more pronounced but it might be expected that the infectivity of such material is also lowered). In conclusion, PCR is a technique that needs further development as it is the only method able to distinguish the origin of the animal species detected in a feed. The developments of kits for the extraction step, initiated by the SAFEED PAP project, should make this phase of the PCR method more standard among the various protocols. PCR should be considered as a confirmatory method of the official method, or as a screening method which would enable light microscopy to more precisely characterise the type of animal by-product detected by PCR, provided microscopic features are present. Other complementarities will be discussed later in this chapter.

A final alternative approach is immunoassays, based on the interaction and specific binding of an antibody (e.g., from a test kit) and an antigen of animal origin present in the feed or ingredient. Different antibodies are used, among them antibodies against troponin I, a major muscle protein that is thermostable, withstands the high-temperature treatment required by EU regulations (method I which is mandatory for mammalian material) and reveals differences in molecular structure according to taxonomic groups. For detecting animal by-products in feed, the most common application of this approach relies on commercial kits intended for screening. The kits are available in ELISA (enzyme linked immunoabsorbant assay) format for laboratory use or in lateral flow format for field use. Lateral flow tests are extremely easy to implement. The rapidity of response and the possibility of automatic readers for interpretion of the results account for the widespread use of this approach in food and feed analysis in the industry. It is nevertheless restricted to screening and never used for confirmatory purposes. Therefore, false positive results are not really a concern, but they do mean that confirmatory methods are required before reaching a final decision on the presence of ruminant by-products. A report by Fumière *et al.* (2008), on the evaluation of Neogen kits for detecting ruminant in feed and MBM which was set up on behalf of the European authorities, revealed that this dipstick test shows some sensitivity problems when the ruminant PAPs are in the presence of PAPs of other animal species. Ruminant PAPs originating from the USA and Australia that had undergone less severe processing than that required by the EU were difficult to detect in PAPs of other animal species, sometimes even when present at levels as high as 30%. The same study presented preliminary results on an ELISA kit commercialised for detecting ruminants in cooked meat and MBM; these results also revealed inconsistencies (mainly false negative results). As the immunological response depends on the heat treatment during PAP processing and generates aberrant results that currently cannot be solved, the use of any immunological method is not suitable for quantitative purposes.

6.5 Detection and quantification of glyceroltriheptanoate (GTH)

According to the recommendations of Commission Regulation EC/1774/2002 for the safe use of animal by-products within the EU and the requirements for the separate and controlled processing and transportation of different categories of animal by-products, there is now an urgent need for a permanent marker to be used for ensuring traceability for disposal (Categories 1 and 2 materials) and eliminating the risk of fraud. Gizzi and von Holst (2002) proposed glyceroltriheptanoate (GTH) as a potential marker. Arguments for the use of GTH were that the molecule is not found in nature; it is commercially available and is already in use as a marker in the food industry; it has a low cost; and it withstands the severe temperature and high pressure treatments used in the EU for rendering animal by-products. Before acceptance as a marker, suitable detection methods for GTH in PAPs had to be developed and validated. The JRC was mandated to do this. The positive results obtained for GTH as a suitable marker for animal by-products led the European Commission to recommend its use for Categories 1 and 2 materials under Commission Regulation EC/1432/2007. The minimal concentration of GTH to use was fixed at 250 mg kg^{-1} related to the fat fraction of the PAPs. The analytical methods for detecting and quantifying GTH are based on both GC/MS and GC with flame ionisation detection (FID). The development and validation studies for these methods have been published by von Holst *et al.* (2009).

The JRC also organised an inter-laboratory study using GC/MS detection and quantification on different samples of marked MBM and fat samples, including blanks (Boix *et al.*, 2010). The results of these studies, from 19 participating laboratories using the GC/MS method, showed adequate values for both relative standard deviation for repeatability (RSD_r ranging from 3.4 to 7.8%) and relative standard deviation for reproducibility (RSD_R ranging from 9.0 to 16.5%). The accuracy of the detected concentration compared with the target concentration was also acceptable, as was the specificity of the identification of unmarked samples. Boix *et al.* (2010) concluded that the method was suitable for the detection and quantification of GTH for official controls. From all this work, the EU now has a strict legal framework for ensuring the reliable traceability of animal by-products classified as unfit for animal feed or human consumption. This legal framework relies on the use of a valuable marker and on validated analytical methods. The implementation of the legislation therefore depends on the efficiency of the control authorities in performance checks and also, as indicated in Commission Regulation EC/1432/2007, on the operators to constantly monitor their processing plants and make this information available to the control authorities. These latter conditions are a pre-requisite for the elimination of the risk of fraud.

6.6 Future analytical methods

As this discussion shows, the official light microscopy-based method, despite some shortcomings, is currently the most satisfactory method for detecting animal by-products in feed. Improvements to this method are possible but limited. What is clearly required is the revision of the legal text in force: work on achieving an optimised standardisation of the equipment and the way the results are reported needs to be carried out. Currently, due to the prescriptions of the zero tolerance policy, there might be a trend to consider a sample as positive when just one particle of animal origin is detected. Such reporting is nonsense from a scientific point of view. Repetitions of analyses under such circumstances will reveal the lack of repeatability of the qualitative analysis. There is therefore a high risk of false positive results with an α-error, or false negative results with a β-error, in basing a result on the observation of a limited number of animal particles. Acceptable risks for these α- and β-errors must be considered when establishing the decision and detection limits (LOD) for analytical methods, as defined in Commission Decision EC/657/2002. But there are no guidelines for establishing the LOD for qualitative methods such as the microscopic method. In this regard, only an accurate way of defining β-errors in qualitative (i.e. binary) results will enable an LOD to be fixed for this method and therefore ensure a results expression with a validated statistical significance and a reliable final result. The EURL-AP is working on this issue. Another issue is the limitation of the microscopic method in identifying the species origin of animal particles found in a feed. The discovery of new microscopic markers that might allow further specification of particles is difficult and therefore the species-to-species feed ban as formulated by the Commission Regulation EC/1774/2002 cannot rely on the sole microscopic method for its future implementation. Improvements in the detection and identification of the origin of animal by-product have to be based on a combined approach (see Fig. 6.2) taking account of the respective advantages of the available methods (PCR, NIRM and immunoassays).

Basically a two-step process is proposed: a screening step and a confirmatory step. Initial detection can be done by applying a screening method (immunoassays, light microscopy, and NIR-based methods – either spectroscopy or microscopy). As no current method is accurate in all respects, any of these methods could be selected for screening provided the confirmation is based on another complementary method, which allows both the detection of components of other chemical composition but also a better taxonomic specification. It is basically a triangulation issue. As an illustration, the disclosure of pig PAP contamination in a pure fish meal batch might be achieved in a first analysis using light microscopy, which will detect terrestrial bone fragments, or using NIRM, which will reveal the presence of terrestrial particles. Confirmation should then be done using PCR with a different taxonomic marker, which would specify the porcine DNA origin

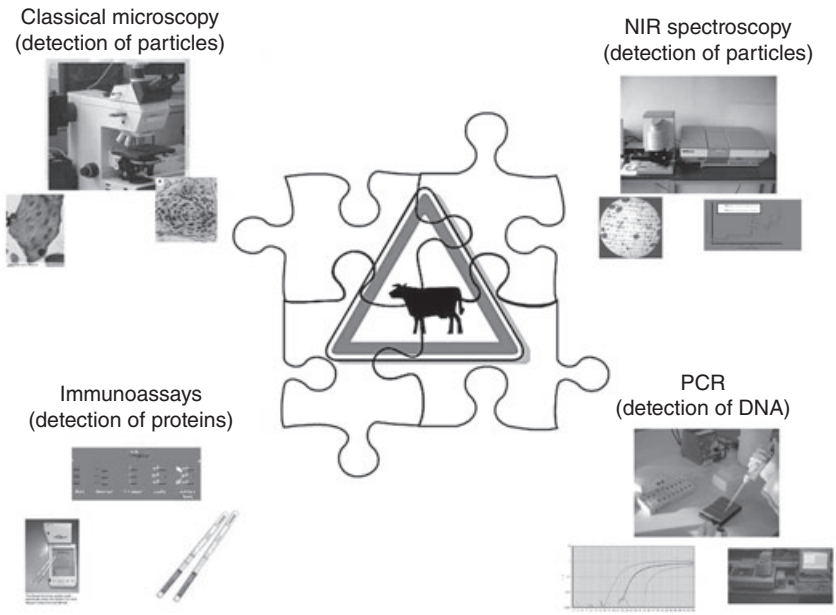

Fig. 6.2 Identification of prohibited animal by-products in feed is like a jigsaw. A final realistic vision on the type of adulteration can only be achieved by applying a multiple method approach.

of the contamination while excluding, by absence of reaction, the possibility of the presence of by-products of other origins (bovine, ovine, poultry, etc.). If screening is done using the non-destructive NIRM, then even the terrestrial particles (either muscle or bone) could be isolated and recovered for subsequent confirmatory analysis using PCR.

With regard to the species-to-species feed ban, the need to develop new molecular markers (DNA targets and antibodies) is crucial. Data collected from official controls within the EU reveal new issues, among them the frequent detection of terrestrial bone particles in fish meal. Putative sources of contamination are sea mammals, dolphins, porpoises and seals, caught accidentally by fishing nets. Whenever such particles are found, the samples are declared as positive for terrestrial animals. Specific markers, either microscopic or DNA-based, for cetaceans and pinnipeds have not yet been developed but should be in the future. Such markers for this order and superfamily will allow a distinction from other mammalian subgroups with regard to the potential risk of TSE transmission.

6.7 Conclusion

The detection of animal by-products, including banned MBM and PAPs, by light microscopy in the EU has considerably reduced the spread of BSE.

The official method under Commission Regulation EC/152/2009 has proved to be efficient, but needs further improvement in order to meet future needs. The potential for improving the method by (1) standardising the equipment, (2) using clearly defined operational sequences and (3) harmonising the reporting of results based on establishing the limits of detection is promising. The development of new microscopic markers is likely, but this development will be limited. Improvements in the detection of banned animal by-products therefore need to rely on a combination of the current methods and alternative methods such as PCR, NIRM or immunoassays. There is no single combinatory solution because many ingredients of different natures are found in feed. For this reason, Fumière *et al.* (2009) suggested the use of an analytical model of the combination of methods to use when animal constituents in a feed have been discovered during an initial first screening. The model relies on the potential of each method to answer questions related to species identification, the authorised or prohibited nature of the detected animal ingredient and the level of contamination. Such analytical approaches, as well as the development of new taxonomic markers, must be in place before applying the species-to-species feed ban which should replace the current extended feed ban.

6.8 Sources of further information and advice

- IAG – International Association for Feedingstuff Analysis – Section Feedingstuff Microscopy: www.iag-micro.org
- European Union Reference Laboratory for animal proteins in feedingstuffs: eurl.craw.eu
- Feed Safety platform: www.feedsafety.org

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7

Hazardous chemicals as animal feed contaminants and methods for their detection

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Abstract: Animal feed can be a route of entry for contaminants into the food chain. Some contaminants, such as dioxins, PCBs and brominated compounds commonly used as flame retardants, bioaccumulate in animal tissues and contribute significantly to human exposure. Many contaminants are subject to legislation, not only in terms of maximum limits prescribed for feed materials and food, but also in terms of analytical methods used for official control purposes.

Key words: chemical contaminants, chemical analysis, official control, standard methods, legislation.

7.1 Introduction

There is an increasing understanding of the importance of the quality of animal feed as a key part of food safety. This has been highlighted in recent years by various worldwide food safety incidents that have all arisen as a result of contaminated animal feed. Many recent dioxin incidents have resulted from the use of contaminated feed combined with the fact that these contaminants bioaccumulate and increase in concentration at higher trophic levels within the food web. The 1999 incident in Belgium involved a PCB mixture being used directly in animal feed, whereas the 2009 incident in Ireland involved the use of PCBs in a fuel source which was directly in contact with feed production. Before either of these was the incident in 1997–1999 in which Brazilian citrus pellets were contaminated with dioxins as a result of the use of contaminated lime in the drying process used in feed, resulting in elevated levels in German milk that were found as a result of surveillance. Another example is melamine, which was found to be added in 2007 to pet food in China to give a false indication of high protein content

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and resulted in several pet fatalities. This is the same compound that was later added to milk that had been diluted in order to give an artificial indication of high protein content, and resulted in several infant fatalities (Gossner *et al.*, 2009; Yang *et al.*, 2009; EFSA, 2010a). As a result of incidents such as these and also due to animal health considerations and the use of chemicals as animal feed additives (both permitted and non-permitted use), regulatory controls in terms of legislation setting maximum limits for contaminants in feed has increased dramatically over recent years and is expected to increase further in future.

Chemical contaminants can arise in animal feed from environmental pollution or as a result of toxins arising from specific primary and secondary metabolites produced by plants used in the production process. Although this chapter deals with chemical contaminants, toxic compounds resulting from either plant or microbial origin share common features, with these chemicals exerting, for example, anti-nutritional effects or reducing reproductive performance or causing some other adverse health effect. It is important not to view these classes of compounds in complete isolation because there is also the possibility of synergistic interactions, although our understanding of the mechanism and magnitude of these effects is still not complete. Incidents involving contaminants and toxins in feed, when they happen, can occur on a global scale, but there is a difference in impact based on the toxicity of the contaminant concerned. Natural toxins (mycotoxins, plant toxins), TSE-related contaminants, microbiological contaminants, and unintended GM materials in feed are dealt with elsewhere in this book. This also applies to trace elements (including heavy metals and arsenic), some pesticides, industrial pollutants and other persistent organic pollutants (POPs) that may contaminate animal feed. This chapter aims to provide a brief introduction to those contaminants likely to pose a significant threat to health, either directly to the animal or to humans as a result of using animal products for food.

7.2 Hazardous chemicals

Many food chemical incidents have an origin in contaminated animal feed that ends up in contaminated animal produce destined for human consumption. Some contaminants, namely persistent lipophilic compounds that bioaccumulate, will increase in concentration (biomagnify) as they move to higher levels of the food chain. Classic examples of this are dioxins and PCBs which have been subject to numerous food incidents, including that in Belgium in 1999 where waste transformer oil was directly incorporated into animal feed, and more recently the incident in Ireland where PCBs were used in fuel used to dry waste bread products that were used as feed ingredients (a more detailed overview is given in the following chapter). In addition to these cases of accidental feed contamination, there have

been cases where feed and food has been deliberately contaminated for economic gain. This includes the use of melamine in China where it was added to increase the total nitrogen content of the milk, giving an apparent high protein reading and thus attracting a higher price for the milk produced. Unfortunately the melamine in the milk had an adverse effect on human health, and the relatively large amount consumed by infants combined with their specific vulnerability meant that there were serious consequences, with many affected babies being hospitalised and even fatal incidents recorded.

7.2.1 Pesticides

Residues of pesticides are more commonly associated with foods of plant origin and it is the use of plants and plant products, such as grain, in the production of animal feed that is usually the source of residues. Organophosphates, pyrethroids and carbamates are relatively quickly degraded and therefore less likely to be found in high concentrations if a long period of time has lapsed after application. Some pesticides are classed as (persistent organic pollutants) POPs and, as such, residues can be found in the environment and can also be present in animal feed ingredients, e.g. cocoa bean husks. For example, the occurrence of organochlorine residues in cows' milk, produced in countries where organochlorine pesticides have not been used for some years, may be attributed to the use of contaminated animal feed or animal feed ingredients imported from mainly less developed countries where they are still in current use. Such pesticides include among others dichlorodiphenyltrichloroethane (DDT), lindane (hexachlorocyclohexane, γ-HCH) and other HCHs, hexachlorobenzene (HCB), aldrin and dieldrin, and chlordane, together with their degradation products and metabolites. In developed countries, the application and use of pesticides is legally controlled to minimise residue levels occurring in both food and feed. Where they are used according to good agricultural practice, residues of these pesticides should not exceed Maximum Residue Levels (MRLs), which are set on the basis of a toxicological risk assessment and in consideration of what is achievable by best practice, i.e. correct application rates and minimum harvest intervals.

Most developed countries have in place monitoring programmes to examine both home-produced and imported animal feed (and food for human use), and whilst the emphasis of these programmes is directed towards products of plant origin, there is still a significant level of monitoring of animal and fish products which are either intended for human consumption or which may be used in pet foods and to some extent (fishmeal and fish oil) in farm animal diets (ACAF, 2009). The number of MRL exceedances in feed in the UK has been reported to be as high as about a fifth of all products, with the most common residue being pirimiphos-methyl, an insecticide used in grain stores (Berry, 2006).

Persistent pesticides may also be found in aquatic systems. They may arise from direct use in wetlands where they may be used to control vector insects, e.g. DDT has been used to control the spread of malaria by mosquitoes, and they may also be used in fish farming, e.g. some organophosphates are used to control sea-lice infections of farmed salmon. Pesticides, especially herbicides, can also enter river systems as a result of rainwater and irrigation wash-off from agricultural land into rivers. There is then a strong potential for these compounds to biomagnify and to accumulate in fish and other aquatic fauna. The residues will then re-enter the land-based food chain if fish or other water-based products (such as seaweed products) are used in animal feed or pet food products. This is particularly true for organochlorine pesticides that are highly lipophilic and can quickly accumulate in oily fish. It is for this reason that oily fish products are being used less and less as feed ingredients, even in the aquaculture industry.

7.2.2 Toxaphene

Toxaphene or camphechlor is a complex mixture of polychlorinated bornanes (CHBs) and other camphenes. Historically, it was one of the most heavily used chlorinated pesticides in the world, with the total quantities used estimated in megatonnes, which is comparable to the usage of polychlorinated biphenyls (PCBs) (see later). It is no longer used and is classed as one of the so-called Stockholm POPs (POPs listed in the Stockholm Convention on Persistent Organic Pollutants, initiated in 2001: www.pops.int). Toxaphene has been shown to undergo long-range transport and is recognised as a ubiquitous environmental contaminant. Like other organochlorine pesticides, it has also been shown to bioaccumulate in aquatic organisms. There is little data on toxaphene in animal feed but it is associated with fish and seafood products. Although toxicological data is scant, toxaphene is a probable carcinogen and is a known endocrine disruptor. Due to weathering and biotransformation, the residue composition in an animal feed product will not necessarily reflect the original pesticide mixtures used.

7.2.3 Dioxins and PCBs

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (collectively referred to as 'dioxins') arise as a result of combustion processes, as by-products in the manufacture of organochlorine compounds or as a result of activity of the chlorine industry. They are chemically stable and are ubiquitously present in the environment even when there is no history of direct occupational or accidental exposure. Although dioxins are present only at very low levels in most plant materials used for animal feed, it is the fact that these chemicals bioaccumulate and biomagnify at higher levels in the food web that makes them so important to control and results

in the requirement for the very low legislative limits that exist in some parts of the world.

Although there are a total of 210 dioxins, it is only the 17 laterally substituted congeners, i.e. those that contain chlorine in the 2, 3, 7 and 8 positions, that persist and accumulate in animal tissues. These congeners form only a small proportion of the total output from many sources and environmental pollution. It is also these 2,3,7,8-substituted congeners that are regarded as significantly toxic and have thus been the main focus of most exposure studies. They are highly lipophilic and are thus found primarily in fatty tissues such as human and animal fats and fish oils.

Polychlorinated biphenyls (PCBs) are a group of compounds which were manufactured until the 1980s for use in various ways including in electrical products, e.g. as a dielectric in transformer oil. They are also ubiquitous environmental pollutants, and it has also become widely accepted that some PCBs elicit dioxin-like biochemical and toxic responses. These are the co-planar PCBs, i.e. those with no or only one *ortho*-substituent. Assessment of the health risks of exposure to dioxin-like chemicals must therefore consider these PCBs in addition to the dioxins. The amount of information pertaining to dioxin-like PCBs in foods is somewhat less than for PCDDs and PCDFs themselves, but is growing rapidly. PCBs have a variety of other biological effects, however, and although consideration of 'dioxins' is incomplete without the inclusion of dioxin-like PCBs, the different types of toxic effects of these and other PCBs should also be taken into account.

Because of their toxicity, both dioxins and dioxin-like PCBs need to be measured at extremely low concentrations in food, and the sum of dioxins and dioxin-like PCBs present is usually expressed in picograms dioxins (as toxic equivalents to the most toxic 2,3,7,8-TCDD) per gram of food. One picogram is 1/1,000,000,000,000 gram. Analysis at these concentrations is extremely technically challenging and expensive and is carried out in only a few specialist facilities worldwide, although demand and capacity for dioxin analysis is increasing fast. The majority of PCB analyses are carried out using gas chromatography using more routine methods, but these often do not measure the dioxin-like PCBs which are present at much lower concentrations in the environment than other congeners.

In addition to the general environmental contamination from dioxins and PCBs, there have been specific isolated events, which have resulted in their release into the environment and hence incorporation into food within a localised area. Such incidents have included the accident in Seveso in 1976 where a manufacturing plant producing a chlorinated herbicide exploded, scattering several kilograms of 2,3,7,8-tetrachlorodibendo-*p*-dioxin (2,3,7,8-TCDD, the most toxic of the dioxins) around the immediate locality of the factory; the spraying of contaminated 'Agent Orange' herbicide (2,4,5-T) in Vietnam in the 1960s conflict in order to defoliate the jungle; the Yusho and Yu-cheng contaminated rice oil incidents in Japan and Taiwan respectively;

and the contamination of animal feed with transformer oil containing PCBs and (to a lesser extent) dioxins in Belgium in 1999.

7.2.4 Tolerable intake

In 1990 WHO established a TDI of 10 pg/kg bw for 2,3,7,8-TCDD, but in 1998 an expert consultation concluded that the TDI should include other dioxins and PCBs which exhibit a similar toxic effect. The concentration of the toxic congeners were weighted according to their toxicity to give units expressed as toxic equivalents (WHO-TEQs) and was established as a range of 1–4 pg WHO-TEQ/kg bw per day. More recently, at the end of May 2001, the Scientific Committee on Food (SCF), an expert committee that advises the European Commission, decided that the tolerable intake should be expressed on a weekly rather than a daily basis and set a tolerable *weekly* intake (TWI) of 14 pg WHO-TEQ/kg bw per week. The WHO/FAO Joint Expert Committee on Food Additives (JECFA) established, in June 2001, a provisional tolerable *monthly* intake (PTMI) of 70 pg WHO-TEQ/kg bw per month. Current estimates of consumer exposure show that intake of dioxins and dioxin-like PCBs is between 1.2 and 3 pg WHO-TEQ/kg bw per day, which is a range that overlaps with the range of the recommended limits. It is therefore important that steps are taken such that the amount of these substances found in food is reduced, by the implementation and enforcement of pollution control measures.

7.2.5 Legislation

Although regulatory limits for dioxins in food have been set on an *ad hoc* basis by various authorities in the past, the EU became the first body to set extensive and comprehensive limits for these compounds. These EU regulations came into force in July 2002, and include limits for PCDDs and PCDFs in food and animal feed. Limits for dioxin-like PCBs were subsequently included (EC, 2006). The regulation was supported by a monitoring plan, and by strict performance criteria for analytical methods that are used. Following a review in 2005 by the WHO on the relative toxicities of the individual dioxin and PCB congeners, and with the results of monitoring and surveillance efforts since the introduction of this legislation, the EU revised these limits taking effect from 2012 (EU, 2011). Recently the CONTAM Panel of EFSA published scientific risk assessments for various POPs in animal feed and in the food chain, as well as statements in dioxins and an opinion on dioxin-like PCBs (www. EFSA. europa.eu).

7.2.6 Concentrations in meat and fish

Because PCDDs, PCDFs and PCBs are lipid soluble, for most food types containing over about 2% fat, concentrations found are reported on a fat-weight basis rather than on a whole product basis. This gives more consistency for comparisons of samples containing variable concentrations of fat

such as dairy products, which show more variability with respect to dioxins on a whole weight basis than on a lipid basis. For some samples, however, reporting on a fat-weight basis may lead to confusion. Fish can show large seasonal variations in fat content, which can result in data reported on a fat-weight basis as having an illusion of variation, even if the body burden with respect to dioxins remains constant.

As pollution control measures are introduced and come into effect, levels of dioxins and PCBs in meat have started to show a downward trend. The same is true, but to a lesser extent, for fish.

Most data available suggest that mean dioxin levels on a fat basis in pork are in most cases lower than for beef, poultry or mutton. Concentrations on a fat basis in animal liver tissue are higher for the same species. Pork typically contains below 0.4 pg I-TEQ/g fat (I-TEQ does not account for the dioxin-like PCBs, which were not measured in many studies) whereas in beef, poultry and mutton the concentration is more typically between 0.6 and 1.0 pg I-TEQ/g fat. If PCBs are included, these concentrations are likely to increase by a factor of about 2.

There is a wide spread of data reported for fish, probably because of the large number of species, and also the geographical differences in the levels of contamination in the various fishing grounds from which the fish originate. Typically, many fish species will contain levels below 1 pg I-TEQ/g but other oily fish species and some shellfish species, especially if sourced from a relatively highly polluted area, will often contain much higher levels. Certain fish species originating from the Baltic region are recognised as containing high concentrations of PCDD/Fs and PCBs. A significant proportion of fatty fish from this region such as Baltic herring and Baltic salmon are unlikely to comply with the EU limit for PCDD/Fs of 4 pg WHO-TEQ/g fresh weight introduced in July 2002, and these fish would therefore be excluded from the Swedish and Finnish diets. There are indications that such an exclusion would have a negative health impact in Sweden and Finland, and consequently there is a local exemption to compliance with the legislation. Sweden and Finland have in place a system which informs consumers about the dietary recommendations concerning the consumption of fish from the Baltic region, in order to avoid possible health risks.

Another source of dioxins in meat can be from pentachlorophenol (PCP) treated wood (for preservative purposes) used to house farm animals and poultry. PCP can contain traces of dioxins in a characteristic congener profile and there have been incidents when this was thought to account for the elevated contaminant levels found in meat.

7.2.7 Brominated compounds

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in soft furnishings and in office equipment, such as computers. They

are POPs and behave in the environment in a similar way to the dioxins and PCBs, and some congeners are thought to exhibit similar modes of toxic action. Polybrominated biphenyls (PBBs) are chemically similar to the PCBs (with chlorine replaced by bromine), and these compounds are still in use as flame retardants and dielectrics. Both PBBs and PBDEs can react to form brominated dioxins, and where there is a source of chlorine, mixed bromine–chlorine analogues may also be formed. Some of these compounds are likely to exhibit similar undesirable effects as the toxic chlorinated dioxins, although as yet there is little data about the presence of PBDD/Fs in food and the environment (there is more data about PBDEs and PBBs, e.g. EFSA (2006) and EFSA (2010b); www.EFSA.europe.eu).

7.2.8 Animal feed as an analytical matrix

The analysis of animal feed cannot be viewed in a simplistic manner. Animal feed can in fact be more variable than food for human consumption. The term includes not only mass-produced composite feed pellets of various types, but also products as diverse as silage, molasses and chicken viscera or other by-products from the production of food for human consumption. In addition to feed for farm animals, pet foods should also be considered and commercially produced pet foods and treats also cover a diverse range of products. This will often mean in analytical terms that a single method is not sufficient. Different extraction techniques may be needed for fatty food types from those used for vegetation.

For many contaminants, validated standard methods exist for various food materials. Animal feed, however, at lower cost and lower risk (at least perceived lower risk to human health), is often not as well validated as a matrix for these contaminants.

7.3 Legislation

European legislation on animal feeds provides a framework for ensuring that feedstuffs do not present any danger to human or animal health, or to the environment. It includes rules on the circulation and use of feed materials, requirements for feed hygiene, rules on undesirable substances in animal feed, legislation on genetically modified food and feed ingredients, and conditions for the use of additives in animal nutrition. Specific legislation on limits and concerning specific types of contamination place demands on analytical methods and performance. All methods used for checking compliance with legislation need to be 'fit for purpose'. This is defined by the Eurachem Guide: *The fitness for purpose of analytical methods: A laboratory guide to method validation and related topics*, 1998, as 'the degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose'.

7.3.1 Official control of animal feed

For analyses undertaken in support of consumer protection in the legal context of 'free trade' (e.g. between WTO members or within the EU) there is a requirement for *agreed* methods of analysis or methods with *agreed* performance. If imported goods are to be rejected on the basis of non-compliance with regulations, there needs to be an *agreement* on how these controls are enforced in different countries and *agreement* that this is done in a uniform manner. Within the EU, there are agreements for free trade, but there are 27 different competent authorities with an even larger number of National Reference Laboratories and many more official control laboratories. Hence there is a need for standardisation of some kind to remove 'instability, fragmentation and overlap'. Therefore EU Directive 85/591/EEC: 'Introduction of Community methods of sampling and analysis' and Regulation 882/2004: 'Official control of foodstuffs' have been introduced to enforce feed and food law, animal health and welfare rules and monitor and verify that the relevant requirements therein are fulfilled by business operators at all stages of production, processing distribution and processing within the EU.

Directive 85/591/EEC concerning the introduction of Community methods of sampling and analysis for the monitoring of foodstuffs intended for human consumption says in the preamble: 'Whereas the *methods of* sampling and *analysis* used for this purpose can have direct repercussions on the establishment and functioning of the common market; whereas they *should,* therefore, *be harmonized* …'. Article 2 goes on to state that 'the introduction of the measures provided for in Article 1 (1) *shall not preclude* Member States *from using other tested and scientifically valid methods* provided that this does not hinder the free movement of products recognized as complying with the rules by virtue of Community methods. However, in the event of differences in the interpretation of results, those obtained by the use of Community methods shall be determinant [italics added for emphasis].'

New technologies and analytical research enable us to measure new and emerging contaminants and other chemicals that may be a threat to the health of animals and consequently (sometimes) the consumer. Once risk assessment confirms the threat, legislation may be enacted to limit the amount of these chemicals present in a product that is sold (see above). In order to enforce this legislation it is necessary to be able to detect the presence of a chemical and measure its concentration. Hence, the consumer protection provided by enforcement depends on how well the measurement method performs. We need to be confident that measurement methods are performing sufficiently well to protect the consumer, without leading to the rejection of large quantities of a product that complies with legislation.

Control laboratories for animal feed are often generalist facilities that are required to offer a wide range of capability, often not only in the

feed sector, and they need to do this whilst under variable and sometimes considerable financial restraint. Similarly the level of technology and analytical expertise may vary between laboratories. Hence, many such laboratories rely on the availability of standard methods from a variety of sources that are easy to implement, are sufficiently robust and have been shown to meet the requirements of legislation.

All EU Member States are required to appoint National Reference Laboratories (NRLs) as a requirement of Regulation EC/882/2004 (EC, 2004). There are NRLs for pesticides and veterinary medicines plus the following five containment areas:

- Mycotoxins
- Heavy metals
- Dioxins (PCDD/Fs) and polychlorinated biphenyls (PCBs)
- Polycyclic aromatic hydrocarbons (PAHs)
- Materials and articles in contact with food.

NRLs work closely with the European Union Reference Laboratories (EU-RLs) appointed by the European Commission and with official national control laboratories (OCLs) within their own country. The role of the NRL includes:

- Providing a channel for communication between the Competent Authority, the EU-RL and national control laboratories
- Advice and representation on contaminants
- Production of standard operating procedures, codes of practice and guidance documents
- Compliance assessment via audit ring trials
- National coordination of EU-RL initiatives.

The aim is to create a more comprehensive, integrated, risk-based, EU-wide, 'farm to fork' approach to official controls and to improve the consistency and effectiveness of controls across the EU and, as a consequence, raise standards of food safety and consumer protection.

7.3.2 Standard methods, criteria based approach and research methods

A number of approaches that can be used to provide confidence include (1) the use of standard methods, (2) the use of analytical criteria that describe the performance of a method, and (3) consideration of 'fitness for purpose' based on measurement uncertainty.

Whilst a standard method can be expensive and time consuming to produce, it can provide a definition of consensus among interested parties and stakeholders (e.g. vendors, buyers, enforcement agencies, academia, etc.) and possibly best practice in the sector to which it is applied; it can support free trade within its domain, and reduce costs associated with instability, fragmentation and overlap in practice and responsibilities.

There is a 'hierarchy' of methods of analysis to be used for official control purposes within the EU. Sampling and analysis methods used in the context of official controls need to comply with relevant Community rules or, (a) if no such rules exist, with internationally recognised rules or protocols, for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation, or, (b) in the absence of the above, with other methods fit for the intended purpose or developed in accordance with scientific protocols.

The practical advantages associated with the use of standard methods are:

- They are generally methods that are based on widely accepted principles with sufficient validation data and proven transferability to other laboratories.
- They give a clear description with all details including calibration and calculation.
- They have been agreed by the interested parties and stakeholders.
- Standard methods are usually designed to use equipment and techniques that can be accessed by as wide a range of laboratories as possible.
- Accreditation bodies would only need to review a standard method once in detail.
- Many standards are available in more than one language (CEN produces standards in English, German and French).
- They are particularly useful if it is necessary to demonstrate to, and gain agreement from, all stakeholders that actions based on the results of analytical tests are a necessary protection for consumers rather than a potential barrier to free trade.
- They are also a starting point for new laboratories, for laboratories involved with a wide range of functions where a variety of analyses are undertaken.

However, there are some disadvantages associated with standardisation and standard methods. For example, the process of converting a good analytical method into a standard method can be laborious. The basis for any method used to enforce food safety regulatory requirements is providing evidence that a method delivers valid results. A newly developed and single-laboratory validated method will then normally be subject to formal validation by collaborative trial, usually organised by the method provider or sometimes by a standards body such as AOAC, CEN or the like using agreed international protocols. The performance data from such an exercise can be used to give a firmer indication of fitness for purpose across a number of laboratories. Valid sets of results from at least eight laboratories are usually required for such a ring trial to give sufficient data to calculate repeatability and reproducibility. The method may then go through a process of being considered, approved and eventually issued as a standard.

The process of converting a method that is considered to have demonstrated sufficiently good performance into as a standard method will usually take at least two years.

An alternative to the use of agreed or prescribed analytical methods is the use of analytical methods with agreed or prescribed performance. There are a number of ways in which the performance of methods may be described, which may be particularly useful for different stakeholders. Broadly, method performance might be described either by using analytical 'criteria' such as those traditionally used by analysts (the criteria approach), or by using measurement uncertainty as applied in analytical chemistry since around the turn of the century (the standard uncertainty approach), or by evaluating the consequences of measurement uncertainty for stakeholders (the uncertainty profile approach).

7.3.3 Exposure

Exposure to a chemical in feed is a function of both the concentration of the chemical within the feed and the amount of that feed the animal consumes. Risk assessment will also take into account hazard characterisation (toxicity). Different feed types may be produced for organic and conventional farming practices, and different supplements and so on may be needed for free-range or outdoor-reared animals compared with those reared using intensive practices with no or limited access to outdoors. When access to outdoors is available to farm animals, this may result in increased exposure to and ingestion of soil, which can be an additional source of exposure to contaminants, often at greater concentrations and more significant as a source than animal feed.

7.4 Future trends

So what does the future hold for the detection and determination of chemical contaminants in feed? Firstly, it is fairly safe to assume that there will be continued testing of feed for known chemicals that are subject to legislation using standard methods. We can also assume that some 'emerging' contaminants will become mainstream, that standard methods will become available and that more will become subject to legislation. Secondly, there is likely to be an increasing trend to profile complex mixtures. This will be driven by improved analytical methods, and as the speed of implementation will depend on cost and availability of instrumentation and methodology. Additionally the ability to identify 'unknown' or 'non-targeted' contaminants may improve with the development of techniques such as metabolomics and as the application of instrumentation such as NMR and TOF-MS becomes more widespread. Understanding and controlling the mechanisms of formation, occurrence, action and fate of contaminants

may lead to a reduction in the number of contamination incidents and therefore improved safety.

7.5 Sources of further information and advice

General information and a detailed description of standard analytical methods are available from the following organisations: AOAC International (http://www.aoac.org/); the International Union of Pure and Applied Chemistry (IUPAC) (http://www.iupac.org/) and the European Committee for Standardization (CEN) (http://www.cen.eu).

The EU regulations are presented by the EU Directorate General for Health and Consumers (DG SANCO) (http://ec.europa.eu/dgs/health_consumer/index_en.htm).

Recent scientific risk assessment, including for many substances a complete exposure assessment of the European population, can be retrieved from the website of the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu), section: CONTAM Panel.

National authorities in general have their own websites, such as for example the UK Food Standards Agency (FSA) (http://www.food.gov.uk) and the Food Safety Authority of Ireland (FSAI) (http://www.fsai.ie).

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8

Animal feed contamination by dioxins, polychlorinated biphenyls (PCBs) and brominated flame retardants

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Abstract: Various incidents with dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs) occurred during the last decades, only in some cases resulting in adverse effects in the exposed animals, in particular chickens. However, these compounds are effectively transferred from feed to milk and eggs and accumulate in meat and liver. This results in exposure of consumers. Since part of the population still exceeds the exposure limit, the EU has set strict limits for feed and food in order to further reduce the exposure. Current levels of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dl-PCBs are declining. However, other persistent compounds have been detected in food, like the brominated flame retardants. This chapter will discuss the issues around these persistent compounds.

Key words: dioxins, PCBs, flame retardants, endocrine disruption, feed and food incidents, exposure.

Note: This chapter is an adapted and revised version of Chapter 15, 'Dioxins, polychlorinated biphenyls and brominated flame retardants', by L. A. P. Hoogenboom, published in *Endocrine-disrupting Chemicals in Food*, ed. I. Shaw, Woodhead Publishing Limited, 2009, ISBN: 978-1-84569-218-6, which was a revised and adapted version of Chapter 20, 'Dioxins and Polychlorinated biphenyls (PCBs)', by L. A. P. Hoogenboom, published in *Pesticide, Veterinary and Other Residues in Food*, ed. D. Watson, Woodhead Publishing Limited, 2004, ISBN: 978-1-85573-734-1.

8.1 Introduction

Environmental contaminants may affect the health of animals and humans when present in feed and food. In particular dioxins and other persistent organic pollutants are a group of compounds that causes great concern. This is based not only on the persistence and accumulation of these compounds

in the food chain, but also on the small to non-existent margin between the actual exposure of humans to these compounds and the toxicological guidelines on safe human intakes. Effects caused by dioxins and related compounds in animals and humans show that they should be regarded as endocrine disruptors. The Ah-receptor, ubiquitously present in mammalian cells but with no obvious physiological function, plays a major role in the effects of dioxins. However, the exact mechanisms behind the effects remain to be elucidated.

Due to various measures, levels of PCBs and dioxins in food and humans have started to decline during the past decades. However, more recently other persistent compounds, in particular brominated flame retardants, have been detected in food and also humans, causing concern about possible adverse effects. Although the environment may contribute to the exposure in particular cases, the major part comes from feed and food. Products of animal origin, including fish, contribute most to the intake. Therefore, it is important to understand the potential carry-over of these compounds from feed to animal-derived products like eggs, milk and meat. This chapter presents the current situation on the toxicity of these compounds, their behaviour in the food chain, various incidents and their sources, tools to detect their presence in feed and food, and current exposure levels.

8.2 Dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs)

8.2.1 Dioxins and PCBs

'Dioxins' is a generic name for two groups of compounds, the polychlorinated dibenzofurans (PCDFs) and the polychlorinated dibenzo-*p*-dioxins (PCDDs) (Fig. 8.1). These compounds are characterized by a planar structure and a varying number of chlorine atoms. In practice the 2,3,7,8-substituted congeners are more or less metabolically resistant and as a result accumulate in biological systems. For this reason the focus is completely on these 17 congeners, seven PCDDs and 10 PCDFs. In addition, it has been shown that 12 out of the 209 PCB congeners may also have a planar structure and resemble dioxins in their stability and biological effects. In particular PCB 126, a so-called non-ortho-PCB, contributes significantly to the overall exposure of humans to dioxins and dioxin-like PCBs.

Sources of dioxins

Dioxins are produced as by-products in the synthesis of certain chemicals, like the wood preserving agent pentachlorophenol (PCP) and the herbicide 2,4,5-trichlorophenoxyacetic acid (Agent Orange). However, they may also be present in heated PCB oil and are formed during incineration of plastics and other waste. Each source may produce its own specific congener pattern.

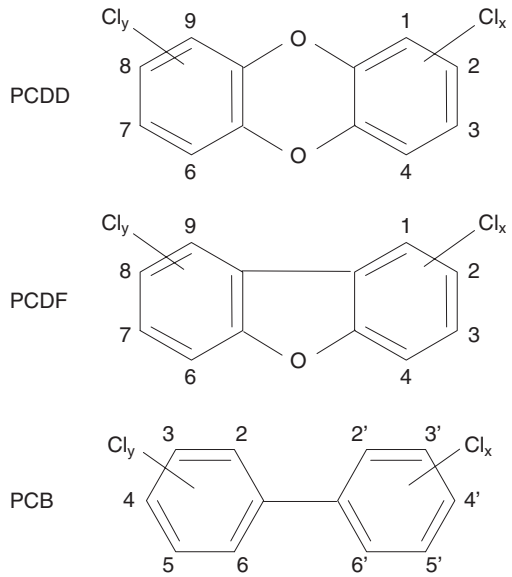

Fig. 8.1 Structures of polychlorinated dibenzo-*p*-dioxins (PCDD), dibenzofurans (PCDF), and polychlorobiphenyls (PCB). The most toxic congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The other toxic six PCDD and 10 PCDF congeners contain at least four chlorine atoms, always at the 2, 3, 7 and 8 positions. The 12 toxic PCBs contain four or more chlorine atoms and none (non-ortho) or only one (mono-ortho) of them is at the 2, 2′, 6 and 6′ position.

Figure 8.2 shows the congener patterns of dioxins in a number of recent food and feed incidents, presented as the relative contribution of each congener to the TEQ level. They are very typical for the source and can actually be used to identify a source.

Sources of PCBs

PCBs are a group of 209 different congeners, which have been produced as technical mixtures with names like Arochlor and Kaneclor. They have been used in large amounts as heat transfer fluids, hydraulic lubricants and dielectric fluids for capacitors and transformers (Safe, 1994). Although these uses are now generally prohibited, the main potential problems are in the disposal of old electrical equipment and the persistence of PCB residues in the environment. Twelve of the PCB congeners have a planar structure and similar properties to dioxins. Other PCBs have been shown to affect brain development (Schmidt, 1999) and appear to be responsible for the tumour promotion effects of these mixtures (van der Plas *et al.*, 2001). In addition, metabolites of non-dioxin-like PCBs interfere with the homeostasis of vitamin A and thyroid hormones (Safe, 1994). In practice, the consumer will be exposed to a mixture of dioxins, dioxin-like PCBs and other PCBs, and

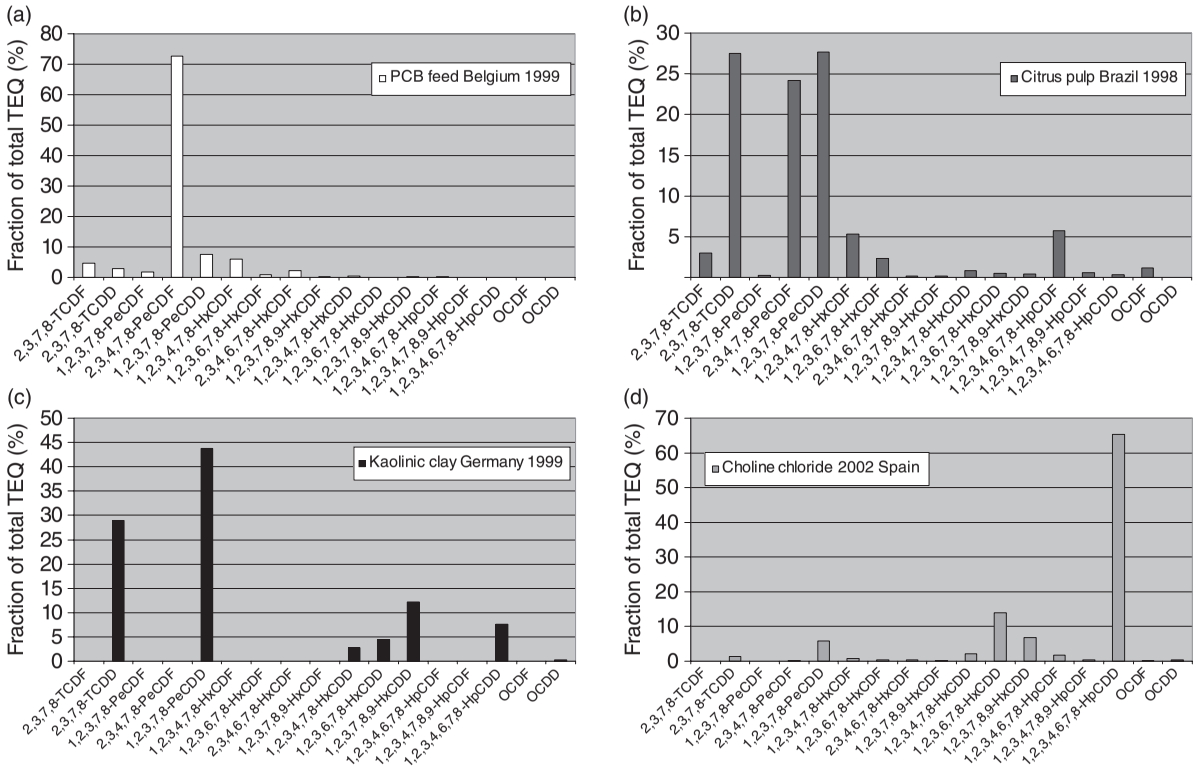

Fig. 8.2 Dioxin congener patterns of a number of contaminations of the food chain, expressed as the relative contribution to the TEQ level (TEFs 1998). Patterns shown are those from (a) the Belgian incident in 1999 (PCB oil), (b) the citrus pulp incident in 1998 (lime from PVC production), (c) the kaolinic clay in 1999, and (d) the choline chloride discovered in Spain in 2002 due to mixing with PCP contaminated sawdust.

the toxicology of these mixtures is very complex. EFSA reviewed the toxicity of non-dioxin-like PCBs and concluded that the most sensitive effects, the tumour promoting effects, are likely to be caused by the dioxin-like compounds in the tested mixtures (EFSA, 2005). As a result, no exposure limits for non-dioxin-like PCBs have been established thus far. However, since most countries set limits for these compounds in the past, the EU decided to establish harmonized food and feed limits that will be in place by the end of 2011. As shown above, elevated levels of PCBs point to the presence of dioxin-like PCBs and PCDFs, although this relationship may vary due to the different PCB mixtures used in the past (EFSA, 2005). As shown by the Irish incident, dioxin levels may be strongly increased by burning of the oil.

In order to avoid difficulties in analysing all 209 congeners, it is customary to analyse only six or seven so-called indicator PCBs that represent the different technical mixtures. These include PCBs 28, 53, 101, 138, 153 and 180 (sum of six) and sometimes PCB 118 (sum of seven), which is also a dioxin-like mono-ortho PCB. It is important to realize that the total amount of PCBs in a technical mixture may be three to four times higher than the amounts of these indicator substances. This relative contribution may change in animal-derived products due to selective absorption, metabolism, carry-over and accumulation of the different congeners. In particular the higher chlorinated PCBs, like 138, 153 and 180, tend to accumulate.

8.2.2 Incidents with dioxins and PCBs

Dioxins were first identified as a possible threat to the food chain during the early 1960s. Large numbers of chickens died from a disease called chicken oedema disease after consuming contaminated feed (Sanger *et al.*, 1958; Schmittle *et al.*, 1958; Higginbotham *et al.*, 1968; Firestone, 1973). The feed contained fat prepared from cowhides that had been treated with chlorinated phenols. It took about 10 years to identify dioxins as the causative factor, which can partly be explained by the fact that these compounds were new to researchers on environmental contaminants, but also by the very low doses that had already led to adverse effects. Chlorinated chemicals containing trace levels of dioxins caused a number of other incidents, including the dramatic incident in Seveso, Italy in 1976. Other examples are the Yu-Sho and Yu-Cheng rice oil contaminations in Japan and Taiwan in 1968 and 1979 respectively, confirming the considerable toxicity of these compounds at relatively low levels. In these incidents, chloracne of the skin was the first overt sign of the exposure of the victims. The two rice oil incidents were caused by leakage of PCB oil, used as heat transfer fluid, into the oil used for human consumption (Kuratsune *et al.*, 1972; Hsu *et al.*, 1985). Only later did it become apparent that the PCB oil contained dioxin-like compounds, like the dioxin-like PCBs but also PCDFs, which might be responsible for most of the observed effects.

In the late 1970s and the 1980s another important source for dioxins was detected: the incineration of municipal waste (Olie *et al*., 1977). In several countries including the Netherlands it was shown that this resulted in elevated levels of dioxins and dl-PCBs in soil and grass and as a result in milk from dairy cows (Liem *et al*., 1991). Levels were too low to result in clear effects in the cows. In most countries, large investments were made to reduce the emission of dioxins from MWIs with the result that dioxin and dl-PCB levels in milk decreased over time. However, soil levels decrease only slowly in such areas and may remain a source of elevated levels in milk and eggs.

More recent incidents with PCBs and dioxins have shown that these compounds still pose a major threat to the food chain. In the US at the end of the 1990s, dioxins were detected in ball clay used in feed for chickens and catfish (Hayward *et al*., 1999; Ferrario *et al*., 2000). In Europe, citrus pulp used for animal feed was shown to be contaminated with PCDDs, resulting in moderately increased levels in milk (Malisch, 2000). The source was the mixing of the pulp with lime from an industrial plant, which turned out to be contaminated with high levels of dioxins. A much larger incident in terms of impact was the chicken crisis in Belgium involving an estimated 200–300 kg PCB oil that contaminated 60 tons of fat used for the production of animal feed (Bernard *et al*., 1999; van Larebeke *et al*., 2001). Again chickens were affected, showing decreased egg hatching and symptoms resembling chicken oedema disease. Follow-up on these effects by one of the feed companies led to the discovery of high levels of dioxins and PCBs by RIKILT, three months after the incident had occurred (Hoogenboom *et al*., 1999). The incident had strong political consequences in Belgium, and in Europe resulted in strict regulations on dioxins (EC, 2001, 2002).

Also as a result of increased monitoring, several novel sources were elucidated, such as kaolinic clay used for mixing of vitamins and minerals into feed (Jobst and Aldag, 2000) and choline chloride mixed with sawdust, contaminated with pentachlorophenol (Llerena *et al*., 2003). Sequestered minerals, prepared from seaweed by a novel process, were shown to contain very high levels. Also drying of materials where products came into contact with the smoke resulted in contaminated grass and bakery waste, dried with waste wood (Hoogenboom *et al*., 2004b). In 2004, potato peels containing contaminated kaolinic clay (Hoogenboom *et al*., 2010) caused the highest dioxin levels in milk ever seen in the Netherlands. The clay was used for sorting the potatoes based on density, but ended up in the peels that were used as feed by two adjacent farms. In 2006, the use of contaminated hydrochloric acid for the production of gelatin from pig bones resulted in levels up to 400 ng TEQ/kg in fat that was used for production of feed (Hoogenboom *et al*., 2007). In 2008, elevated dioxin levels were detected by South Korea in pork imported from Chile (Kim *et al*., 2011). The incident caused the closure of the borders for many months. The incident was traced back

to the use of contaminated zinc oxide, produced by a recycling process. A similar incident with zinc oxide from Turkey had occurred in 2005 in Italy, causing increased levels in feed and milk. In all these cases the incidents started in products used as animal feed and were traced back to recycling of fat or other ingredients, the drying with improper fuels or the occurrence of dioxins in certain types of clay.

By the end of 2008, a sample of pig meat was analysed by a French private company and showed a dioxin level of more than 400 pg TEQ/g fat, i.e. 400 times the EU limit of 1 pg TEQ/g fat. The meat was obtained from a company in the Netherlands but was actually traced back to Ireland (Heres *et al*., 2010). It turned out that pigs and cows had been fed with feed containing bakery waste that was dried with an oil containing PCBs. The feed contained primarily PCDFs and also some PCBs, but relatively less than during the Belgian incident, probably due to the burning process.

Increased monitoring in the Netherlands also resulted in the discovery that eggs from laying hens foraging outside may contain elevated levels of dioxins and dl-PCBs. Highest levels observed were around 10–15 pg TEQ/g fat, compared to a limit of 3 pg TEQ/g fat for dioxins and 6 pg TEQ/g for the sum of dioxins and dl-PCBs. Follow-up studies showed that contaminated soil must be the source and that even low background levels may already cause problems. Studies in other countries showed that the issue is not specific to the Netherlands. A number of measures were proposed to reduce the intake of soil and as such the levels (Kijlstra *et al*., 2007). Early in 2010, elevated dioxin levels were again detected in organic eggs in both the Netherlands and Germany. In this case the source was not the soil but the use of corn contaminated with dioxins and PCBs. The source of the incident was not discovered, although there were clear similarities with the Irish incident, based on the ratio of PCBs to dioxins.

At the end of 2010, a German feed company discovered slightly elevated dioxin levels in their feed. The source turned out to be technical fatty acids produced as a by-product of biodiesel production and not intended for feed production but for the paper industry. The fatty acids were shown to contain also tri-, tetra- and pentachlorinated phenols which are known precursors for PCDD/Fs (W. A. Traag, personal communication). The source of the dioxins and chlorophenols is still under investigation.

In addition to feed and soil, housing may also be a potential source of contamination. Brambilla *et al*. (2009) described a case where the use of shavings derived from pentachlorophenol-treated wood caused the contamination of eggs. The issue had already been described in the 1980s by Ryan *et al*. (1985), showing a relation between the contamination of chicken meat with PCP and higher chlorinated PCDD/Fs due to contaminated wood shavings. Another incident occurred within an experimental unit of USDA, causing the contamination of calves in a carry-over study with dioxins (Feil *et al*., 2000). The wood in the stable turned out to be contaminated with pentachlorophenol, causing a background of

highly chlorinated PCDD/Fs in not only the dioxin-treated but also the control calves.

8.2.3 Assessing the toxic effects of dioxins and dioxin-like PCBs

Effects in humans and animals

In laboratory animals, exposure to TCDD results in liver tumours in female rats. Lower levels of exposure result in effects on the immune and reproductive systems, as well as impaired learning (WHO, 2000). Another typical effect is endometriosis, a symptom where cells from the endometrium grow in sites outside the uterine cavity. This effect was first observed in rhesus monkeys exposed for 4 years to doses of 5 and 25 ng TCDD/kg in the diet (Rier *et al*., 1993). The effect only became apparent after an additional 10 years on clean feed. Effects observed in humans exposed accidentally to these compounds are chloracne (following exposure to high concentrations), neurodevelopmental delays in children and neurobehavioural effects, an increased risk of diabetes and an increased risk for certain cancer types like soft tissue sarcomas and liver cancer (WHO, 2000).

Dioxins and dioxin-like PCBs have endocrine-disrupting properties. They bind to the so-called Ah-receptor present in mammalian cells, thus resulting in the transcription of a large number of genes. These genes include those encoding for biotransformation enzymes like cytochromes P450 1A1, 1A2 and 1B1, UDP-glucuronyl-transferase and glutathione-S-transferase 2α, as well as genes involved in the growth regulation of cells (Guo *et al*., 2004). From studies with knock-out animals, it is clear that the Ah-receptor plays a major role in the adverse effects of dioxins. Despite the fact that the Ah receptor regulates the expression of many of the mentioned biotransformation enzymes, the exact mechanisms and role of this receptor remain to be elucidated.

The increased activity of several biotransformation enzymes alters the metabolism and excretion of certain hormones. This results in, for example, decreased thyroid hormone levels (Giacomini *et al*., 2006). The effect is further exaggerated by the competition between dioxins and PCBs, and the thyroid hormones T3 and T4 for the carrier protein transthyretin (TTR). As a result the levels of bound hormones decrease and the degradation of the hormone is further increased. Feed back regulation results in elevated levels of TSH and subsequently effects on the thyroid gland, including tumours (Knerr and Schrenk, 2006). TTR is also involved in the transport of retinoids and TCDD has been shown to interfere also with the homeostasis of these compounds (Murphy *et al*., 2007). Brouillette and Quirion (2008) showed, for example, that TCDD causes a deficit in the memory function of female mice, which could be counteracted by the addition of retinoic acid, but also estradiol. Other studies showed a direct effect on the sex organs. In sex glands of male and female rats, TCDD causes decreased levels of 17 α-hydroxylase/17,20-lyase cytochrome P450 expression, most

likely due to an interference with the effect of hormones like hCG and LH (Moran *et al*., 2003; Fukuzawa *et al*., 2004). The latter study actually showed decreased testosterone levels in the testes of 12-week-old mice, an effect that was not observed in Ah-receptor knock-outs. As a result, the production of androgens and estrogens is decreased, resulting in lower plasma levels. However, at lower TCDD doses Haavisto *et al*. (2001) observed an increased testosterone production in young male rats exposed *in utero*, indicating that timing and dose are very important determinants in the actual effects. Decreased levels of thyroid hormones may also play a role in the effect on testosterone production by the testes, since hypothyroidism is known to affect the Leydig cells in the testes.

Exposure of rats and mice to TCDD results in tumours in liver, thyroid, lung, skin, oral cavity and other sites (Knerr and Schrenk, 2006). Observations that liver tumours primarily occur in female rats have suggested a role of estrogens in this effect, possible through the increased formation of reactive catechol-estrogens (Knerr and Schrenk, 2006). In particular cytochrome P450 1B1 could play an important role in this process. The interaction between estrogens and dioxins is complex and appears to occur also at various other levels. This interaction requires further studies.

Another important issue is the possibly long-lasting effects of perinatal exposure to dioxins. Brown *et al*. (1999) observed that *in utero* exposure of rats to TCDD resulted in a higher incidence of DMBA-induced mammary tumours in adult rats, suggesting an imprinting effect of TCDD. Fenton *et al*., (2002) observed persistent changes in mammary gland development of rats following *in utero* exposure to TCDD, which points in the same direction. In a more recent study by this group (Wang *et al*., 2011), a delayed formation of DMBA-induced mammary tumours in adult mice was observed which could not be related to increased adduct formation or metabolism of DMBA. Nayyar *et al*. (2007) observed in mice exposed *in utero* a similar uterine phenotype at adult age as observed in women with endometriosis.

Also the immune system is an important target for dioxins, since TCDD has been shown to decrease the immune response. Again the exact mechanism remains to be resolved.

The relevance of the effects observed in animals for humans, especially those exposed to low levels of dioxins and PCBs, remains to be shown. TTR, for example, is not as important in humans as in rodents, since thyroid binding globulin (TBG) is the more important carrier protein for thyroid hormones. TCDD has only a weak affinity for TBG. However, the vast amount of data clearly points to the possibility that exposure to dioxins results in a number of subtle effects related to disturbance of hormonal processes.

Exposure limits for dioxins and dl-PCBs

Based on the effects in animals, the World Health Organization (WHO) and the EU Scientific Committee on Food (SCF) have set very low

exposure limits, being respectively a TDI of 2 pg TEQ/kg bw/day (WHO, 2000) and a TWI of 14 pg TEQ/kg bw/week (SCF, 2001). The SCF based the limit on a reduced sperm count of male rats exposed to TCDD *in utero* (Faqi *et al*., 1998). A similar effect was observed in several previous studies but could not be reproduced in more recent studies (Bell *et al*., 2007a, b). However, in the latter study, repeated dosing of the mother resulted in a delayed puberty in the male pups, even in the lowest dose group. This effect was previously observed in other studies. The effects on embryos seem to result from exposure during a rather short critical window of exposure, in rats being around gestational day 15. However, there is still dispute whether adverse effects observed in young animals and humans may also result from exposure early in life, due to exposure through lactation.

Overall, it is clear that the levels in the body that are required for a number of adverse effects are in a similar range. In deriving the exposure limit, WHO and SCF took into account the levels in the various tissues (internal dose or body burden) but also kinetic differences between rats and humans. The expression of limits on a weekly basis acknowledges the fact that critical levels in the body of consumers are unlikely to be reached after a single exposure, but merely after the continuous intake of these compounds at relatively low levels in the food. So the toxicity of dioxins and PCBs is thought to be jointly due to their effects at low levels, their lipophilic nature and their resistance to metabolic degradation, resulting in accumulation in the body. Eventually this may result in body burdens that are higher than safe levels, thus resulting in activation of the Ah-receptor pathway and the adverse effects described above. The exposure limit should prevent body burden levels from eventually reaching levels that may cause adverse effects, especially in pregnant women.

However, animals and humans may be incidentally exposed to very high levels through feed or food. In rats it appears that such a high exposure results in a relatively high transfer of dioxins to the fetus (Hurst *et al*., 2000a, b; Bell *et al*., 2007c). This can be explained by the fact that the fetus can be regarded as a highly perfused tissue, contrary to the fat tissue. However, Bell *et al*. (2007a, b, c) showed that the effects of repeated exposure with respect to delayed puberty seem larger than after a much higher single exposure. It was argued that the acute exposure might have missed a critical window earlier during gestation. This issue requires further investigation, especially regarding the relevance of a peak exposure during certain incidents or following the consumption of, e.g., heavily contaminated fish or clay.

The TEQ principle

The exposure limit was based on effects of the most toxic dioxin TCDD in rats. In practice, however, the exposure limits include all 17 toxic 2,3,7,8-chlorinated PCDDs and PCDFs, as well as 12 planar PCBs. This is

also true for the food and feed limits set by the EU. Dioxin limits were set in 2001. The dioxin-like PCBs were included in November 2006 (EC 2006a, b). Levels of dioxins and dioxin-like PCBs are normally expressed in TEQs (Toxic Equivalents). This refers to the use of the so-called TEQ principle.

Most of the toxicological data have been obtained from studies with TCDD. Most other dioxins and dioxin-like PCBs are not equally toxic, partly due to different kinetics. At the same time there is strong evidence that the effects of the different congeners are additive. In order to deal with this particular issue, the so-called TEQ approach has been introduced. The different congeners have been assigned a so-called TEF value, which is a weighted value, based on the differential effects of different congeners in various experiments. TEF values are consensus factors agreed upon by international scientists involved in the field. The last time these TEF values were evaluated was during a WHO workshop in 2005. These values are shown in Table 8.1, together with the previous set published in 1998 (Van den Berg *et al.*, 1998, 2006). In principle, a compound should have the following properties to obtain a TEF value:

- It must show a structural relationship to the PCDDs and PCDFs.
- It must bind to the Ah receptor.
- It must elicit Ah receptor-mediated biochemical and toxic responses.
- It must be persistent and accumulate in the food chain.

Many other compounds have been shown to have affinity for the Ah receptor, including polyaromatic hydrocarbons and a number of secondary plant metabolites, like furocoumarins. However, these compounds failed to pass the other criteria, primarily due to their rapid degradation in the body. Other compounds like the polybrominated dioxins and biphenyls might be considered for a TEF value, but adequate studies are missing for the assignment of a TEF value.

An important issue is whether the effects of different dioxins and dioxin-like PCBs, but also other Ah receptor agonists, are actually additive or whether there may also be antagonistic activity. Furthermore, Safe (1995, 1998) was one of the first to point out the often very high levels of Ah receptor agonists occurring in certain foods of plant origin, which may be consumed on a frequent base (furocoumarins, indole-3-carbinol). However, there is a substantial difference between these compounds and the relevant dioxins and PCBs, since these plant-derived compounds are often metabolized completely before entering the blood circulation. Further studies are required to reveal which naturally occurring compounds may actually retain their activity in the body. In addition, certain toxic effects of dioxins may be due to the increase in body burden above a certain threshold, subsequently resulting in a continuous stimulation of the Ah receptor pathway. It remains to be elucidated whether natural Ah receptor agonist may reach effective concentrations and cause the related effects.

Table 8.1 TEF values as assigned by the WHO in 1998 and 2005

Compound	TEFs 1998	TEFs 2005
PCDDs		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
OCDD	0.0001	**0.0003**
PCDFs		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PeCDF	0.05	**0.03**
2,3,4,7,8-PeCDF	0.5	**0.3**
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01
OCDF	0.0001	**0.0003**
Non-ortho PCBs		
3,4–3′4′ PCB (77)	0.0001	0.0001
3,4,5–4′ TeCB (81)	0.0001	**0.0003**
3,4–3′4′5′ PCB (126)	0.1	0.1
3,4,5–3′4′5′ PCB (169)	0.01	0.03
Mono-ortho PCBs		
2,3,4–3′,4′ PeCB (105)	0.0001	**0.00003**
2,3,4,5–4′ PeCB (114)	0.0005	**0.00003**
2,4,5–3′,4′ PeCB (118)	0.0001	**0.00003**
3,4,5–2′,4′ PeCB (123)	0.0001	**0.00003**
2,3,4,5–3′,4′ HxCB (156)	0.0005	**0.00003**
2,3,4–3′,4′,5′ HxCB (157)	0.0005	**0.00003**
2,4,5–3′,4′,5′ HxCB (167)	0.00001	**0.00003**
2,3,4,5–3′,4′,5′ HpCB (189)	0.0001	**0.00003**

Values in bold indicate the changes.

It was agreed to evaluate the TEF values every five years, depending on new data from toxicological studies. As a result the TEQ levels in food may change considerably if the TEF value of a congener that contributes significantly is changed. The last revision of the TEFs in practice results in a decrease of the levels of about 15% (Van den Berg *et al.*, 2006), but food items containing high levels of mono-ortho PCBs may show an even larger decrease. Since the EU based its food and feed limits on the background levels determined with the 1998 TEFs, these old TEFs were used in the EU for determining the TEQ levels until the introduction of new food and feed limits in 2012 (EC 2006a, b).

8.3 Exposure to dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs)

8.3.1 Current exposure to dioxins and PCBs

Several studies have shown that at present, the exposure of part of the population in Western countries to dioxins and dioxin-like compounds exceeds the exposure limits set by the WHO and SCF (Liem *et al*., 2000). A study in the Netherlands showed that the median of the lifelong intake of dioxins and dioxin-like PCBs by the Dutch population around 1999 was 1.2 pg TEQ/kg bw. Dioxins and PCBs contributed to a similar extent to the exposure. The 90th percentile was 1.9 pg TEQ/kg bw and it was estimated that 8% of the population exceeded the existing exposure limits (Baars *et al*., 2004). These intakes should be compared to the exposure limit (TWI) of 14 pg TEQ/kg bw/week or on average 2 pg TEQ/kg bw/day. In the same study, the median and 90th percentile for the estimated intake figures for indicator PCBs were respectively 5.6 and 10.4 ng/kg bw/day. Dairy products, meat, oils and fats, fish and vegetable products contributed 27, 23, 17, 16 and 13% respectively to the intake of dioxin-like compounds. Similar figures were obtained for the indicator PCBs, although fish showed a 10% higher contribution. Slightly higher intake levels for dioxins only were obtained by Llobet *et al*. (2003) for the Catalonia region in Spain, showing an average intake of 1.4 pg TEQ/kg bw/day. The higher intake might be explained by the higher consumption of fish products that contributed 31% to the intake. In a study from Japan the relative contribution of fish products was even higher, being more than 50% of the average daily intake of respectively 1.6 and 3.2 pg TEQ/kg bw/day for dioxins and the sum of dioxins and dioxin-like PCBs (Tsutsumi *et al*., 2001).

It is important to note that in general the intake levels have decreased during the last decades, due to various measures to control the major sources. In the Netherlands the intake of dioxins and dioxin-like PCBs decreased from about 8 and 4 pg TEQ/kg bw/day in 1978 and 1984 to respectively 2.2 and 1.2 TEQ/kg bw/day in 1990 and 1999 (Baars *et al*., 2004). This decreased intake is also reflected in the levels detected in human milk, which also showed a marked decrease in the various countries. Apart from incidental high exposures, the major impact of incidents like those in Belgium in 1999 and Ireland in 2008 is probably that these efforts are temporarily diminished.

Consumers with certain preferences may have an increased intake of dioxins and PCBs. It has been shown, for example, that eels from contaminated rivers may contain relatively high levels of dioxins and dioxin-like PCBs (Hoogenboom *et al*., 2006a; van Leeuwen *et al*., 2007). As a result the frequent consumption of such eels will result in elevated exposure. The same may be true for people consuming eggs from free-range chickens, especially when the hens spend a great deal of their time outside (Kijlstra *et al*., 2007). These eggs may contain levels up to 10 pg TEQ/g fat or more

and therefore contribute to a significant exposure. Following the discovery of this problem, most farms were able to reduce these levels by a number of measures, but for private owners this may not be the case. A rather typical case is the use of certain clays during pregnancy by women of African origin, since some of these clays were shown to contain high levels of dioxins. In view of the sensitivity of the unborn child for dioxins, this issue deserves more attention.

8.3.2 Limits in food and feed

In order to further reduce the intake of the population, the EU has developed a strategy for further reducing the exposure. This includes the establishment of maximum levels for dioxins in food products based on the principle 'strict but feasible'. Limits are based on reported levels in various products and as a result differ per product: 1 pg TEQ/g fat for pork, 2 pg TEQ/g fat for poultry, and 3 pg TEQ/g fat for beef, milk and eggs (EC, 2001, 2006a). In addition limits have been set for animal feed (0.75 ng TEQ/kg) and their ingredients (0.75–6 ng/kg TEQ) (EC, 2002, 2006b). Since November 2006, planar dioxin-like PCBs are included in the EU food and feed limits, but the separate limits for dioxins have been maintained. As a result there are limits for dioxins and for the sum of dioxins and dl-PCBs for each food and feed item. In addition, the EU has set action levels for dioxins and dl-PCBs, being about 60–70% of the maximum levels. Samples exceeding the action level should be followed up to determine the source of the pollution. Eventually, this approach should result in a more rapid decrease of the levels and guarantee that no consumers exceed the exposure limit or TWI of 14 pg TEQ/kg bw/week. Limits for food and feed were slightly changed in 2012 primarily because of the use of the TEFs 2005 but not a decrease in the levels in the last 10 years.

8.3.3 Analytical methods for dioxins and PCBs

Because of the low limits for food and feed, dioxins can at present only be analysed by high resolution GC/HRMS. In addition, an extensive clean-up is required, starting with the extraction of fat and its subsequent removal by gel permeation chromatography (GPC) or acid silica. Pesticides and other lipophilic substances need to be removed by various clean-up steps. Eventually dioxins and PCBs are separated on an activated carbon column. In order to account for possible recovery losses in the different column and evaporation steps, ^{13}C-labelled standards are added at the first step. Individual dioxins are identified by retention time and molecular mass, and quantified. Using the TEF values, the levels are transferred into TEQs and summed into figures for dioxins, dioxin-like PCBs and the total sum. The levels are normally reported as lower bound and/or upper bound levels, thereby dealing with the levels of non-detected congeners. When reported

as upper bound levels, as required by current legislation in the EU, the levels of non-detected congeners are set equal to the detection limits.

The intensive clean-up and associated equipment is very costly. Furthermore, sample throughput is relatively small, causing further problems during crisis situations such as during the Belgian dioxin crisis in 1999. The whole clean-up procedure has now been automated, thus eliminating the removal of the solvents between each step. Nevertheless, there is a major need for cheap and high-throughput screening methods that can be used to select potentially contaminated samples and, equally important, to clear negative samples. Initial assays were based on the induction of cytochrome P450 enzymes in hepatoma cells or freshly isolated hepatocytes. These cells contain the different steps of the Ah receptor pathway. Elevated levels of these enzymes are detected by the increased metabolism of ethoxyresorufin into resorufin (EROD activity). However, the assay is potentially sensitive for false-negative results, since the enzyme has a number of inhibitors, although in practice this will depend on whether such compounds will end up in the final extract. Nevertheless, novel assays, like the Chemical Activated Luciferase gene eXpression or CALUX assay, were developed based on cells with reporter genes like that encoding for the firefly enzyme luciferase. Following exposure to dioxin-like compounds, the cells will synthesize luciferase, which can easily be detected by a light-producing assay. Compared to GC/HRMS, the assay requires only a very simple clean-up, usually based on acid silica. However, the assay does not allow the use of internal standards. As a result the clean-up must be simple and highly reproducible, and proper control samples must be included in each test series to control for recovery and possible contaminants in the chemicals used for clean-up. Under these conditions the assay has proven its usefulness for controlling dioxins (Hoogenboom, 2002; Hoogenboom *et al.*, 2004b, 2007). In a regulation, the EU has set guidelines for the performance of analytical methods within a laboratory, including screening assays. GC/HRMS was established as the reference method. Screening methods can be used to eliminate negative samples if the fraction of false-negatives remains below 1%. Suspected samples need to be confirmed by GC/HRMS.

The analysis of the so-called indicator PCBs and, in fact, also the mono-ortho PCBs does not require the same sensitivity due to the much higher levels. In practice, GC with either ECD or MS/MS detection is used. As a result this analysis is much cheaper and more analytical capacity is in general available. In specific cases, like during the Belgian dioxin/PCB crisis in 1999 and possibly also in the case of, for example, eels from polluted rivers, indicator PCB analysis might actually be used as a screening tool for samples (Hoogenboom *et al.*, 2006a). The major problem is that this might result in a more general application and that dioxins from other non-PCB like sources might be overlooked.

8.4 Carry-over of dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs) in food-producing animals

The health risk caused by the chronic exposure to low levels of dioxins and PCBs is partly due to their lipophilic nature and resistance to metabolic degradation, resulting in accumulation in the body. Eventually this may result in body burdens that are higher than critical levels, thus resulting in the adverse effects described above. One of the few mechanisms leading to a significant reduction in body burden levels is the excretion of these compounds in breast milk. Similar is true for food-producing animals, where dioxins are transferred into milk and eggs.

The studies described in this section will show that the carry-over of contaminants from feed to edible products can be influenced by many different factors. This is based on a previous review on the carry-over of persistent contaminants (Hoogenboom, 2005). The process starts with the absorption of the compounds, which is dependent on the physical properties (e.g. lipophilicity) but also the source of the contaminants. Compounds bound to air particles and deposited as such on feed may be differently absorbed than compounds present in fat ingredients in the feed. In the case of dioxins, PCBs, PBBs but also PAHs, it appears that the higher chlorinated and more lipophilic congeners are more poorly absorbed than the lower chlorinated congeners. At the same time, the toxic 2,3,7,8-substituted dioxin congeners seem to be less well absorbed than the less toxic congeners (McLachlan *et al.*, 1990). It should be pointed out that this may also indicate a more efficient excretion of the toxic congeners into the bile. This process is normally not taken into account when determining the bioavailability of compounds. However, studies with PBBs in lactating cows indicate that these compounds are actively excreted into the bile and subsequently the feces (Cook *et al.*, 1978). A very interesting observation is the possible formation of the octa- and possibly hepta-CDD in the GI-tract or feces of cows (Fries *et al.*, 2002b), although the significance with respect to the consumer might be low.

Although many polyhalogenated contaminants are very persistent, metabolism is a second important factor in the carry-over. Many PCBs and even a number of the toxic dioxins appear to be metabolized in food-producing animals, thus explaining their poor recoveries in mass-balance studies. Whether metabolites are present in edible products has not been examined.

Last but not least is the storage or excretion into edible products. Both the carry-over into milk and eggs, as well as the accumulation in body fat or other tissues, depend on the physical properties of the compounds. In the case of dioxins, the carry-over of the lower chlorinated and less lipophilic congeners is higher than that of the higher chlorinated congeners, whereas the non-toxic analogues are not excreted in milk at all. The higher chlorinated compounds tend to accumulate in the liver. Especially at prolonged

exposure, distribution plays an important role. In both laying hens and dairy cows, lipophilic compounds are divided over the body fat and the fat in eggs and milk. The difference is that compounds in milk and eggs are excreted but those in body fat are mobilized again and eventually also excreted in milk and eggs. As a result levels in milk and eggs will increase following repeated exposure and this will result in a steady-state situation where an apparent balance is obtained between the input through the feed and the output into the milk or eggs. For some compounds this situation is reached much earlier than for other compounds. In the case of growing animals like calves, pigs and broilers, the fat compartment will continuously increase and such steady-state conditions will not be obtained. Following absorption, compounds initially distribute to the highly perfused tissues like muscles and liver, as shown by Thorpe *et al.* (2001), who observed that especially during the first days of an elevated exposure, the levels in body fat may underestimate the levels in the fat in the meat.

Following termination of the exposure, the compounds stored in the various body parts become the sole source for transfer of contaminants to the milk or eggs. In general the depletion of persistent dioxins, PCBs and PBBs is characterized by two phases, a rapid initial (distribution) phase and a much slower second (elimination) phase. The first phase can be explained by the relatively rapid excretion of contaminants present in the GI tract and blood. The second part represents the much slower mobilization of contaminants from body fat and liver. It is important to realize that levels in both milk and eggs normally decrease by more than 50% during this first phase. In eggs there is, however, a delay in the start of the elimination phase due to the fact that the production of an egg requires about 10 days. In growing animals, dilution by increasing fat volumes is very important.

The carry-over of persistent contaminants has been modelled, in particular in the lactating cow. Novel approaches are based on pharmacokinetic modelling (PB-PK-modelling) and ideally take into account the size of the different tissues and organs and factors like blood flow and milk production (Derks *et al.*, 1994). Also a number of parameters have been introduced to describe the behaviour. The bioconcentration factor (BCF) is used as the ratio of the concentrations in feed and milk, based on either wet weight or lipid base. This factor is influenced by the amount and feed consumed and as such by factors like water content and energetic value. Therefore, the biotransfer factor (BTF), defined as the ratio of the concentration in the milk and the total ingested quantity of contaminant, seems to be more appropriate, where the concentration in milk is ideally expressed on a fat base. An even more useful parameter is the carry-over rate (COR) defined as the percentage of the total amount ingested which is excreted into the milk or eggs. Ideally this is based on the daily intake and excretion when steady-state conditions are reached, but in the absence of such conditions total intake and (extrapolated) excretion during the study period are also

used (Table 8.2). Sometimes the carry-over rate is used as a synonym of bioavailability, being the fraction of the contaminant absorbed in the GI tract. This does not account for the fact that part of the compounds are excreted in the bile and feces, or metabolized after mobilization from the body fat. Carry-over rates can be used to determine the impact of a certain feed contamination on, e.g., levels in milk. However, as long as there is no equilibrium between intake and output, as during the first days or weeks of exposure, this will lead to an overestimation of the actual milk or egg levels. This is due to the fact that during this period mobilization of the contaminants stored in the liver and body fat and thus their contribution to the milk and egg levels is still rather small.

It has become customary in current regulations for environmental contaminants to focus on the sum of a number of congeners, if possible after applying a toxic equivalency principle. For toxicological reasons this is a very good approach since it takes into account the additive nature of mixtures of compounds. However, when trying to model the behaviour of contaminants in animals it is better to base this on individual congeners regarding the differences in the behaviour described in this chapter. A feed contamination of lower chlorinated dioxins (e.g. as in 2,4,5-T) will give much higher TEQ levels in milk than a feed contamination with a similar TEQ level but derived from higher chlorinated compounds (as in PCP). Similar is true for PCB mixtures, although the variations in congener patterns found in practice might be much lower. However, a rendered fat derived from exposed animals might contain the more stable congeners and as such give a much higher carry-over.

8.4.1 Transfer of PCBs in lactating cows

In 1970, shortly after the Yusho rice oil incident, PCBs were detected for the first time in milk in the eastern part of the US. The source was shown to be feed silos coated with the Arochlor 1254-containing product Cumar (Willett and Hess, 1975). Levels in silage near the coated wall varied between 10 and 60 mg/kg, with occasionally levels over 7 g/kg. A survey revealed that about 7% of the milk samples showed detectable levels of PCBs, but only a few exceeded the existing limit of 2.5 mg/kg fat. This discovery triggered studies on the relationship between PCBs in feed and milk, in order to allow management of the risks evolving from this type of contamination. Fries *et al*. (1973) treated nine cows with 200 mg Arochlor 1254 for 60 days followed by a withdrawal period on clean feed for another 60 days. Individual PCBs could not be identified at that time. PCB levels in milk increased rapidly and a steady state was reached after 40 days with average PCB levels in milk of 61 mg/kg fat. Concentrations in body fat, determined in fat biopsy samples obtained after 30 and 60 days of exposure, were respectively 20 and 42 mg/kg. During the elimination period levels in milk fat declined 50% within 15 days, but then slowed down. Levels in body fat decreased

Table 8.2 Carry-over rates (%) as determined in lactating cows

	Firestone *et al.* (1979)[1]	McLachlan *et al.* (1990)[1]	Olling *et al.* (1991)[2]	Slob *et al.* (1995)[1]	Schuler *et al.* (1997a)	McLachlan and Richter (1998)[1]		Fries *et al.* (1999)[1]	Malisch (2000)[1]
Daily dose (ng TEQ/kg bw/day)	36.4	0.012	11.1	0.20	0.014	0.002	0.018	0.11	0.02
Source	PCP	Natural	Standards	MWI	MWI	Natural	Sludge	PCP	Citrus pulp
Number of cows	3	1	4	Many	41	4	4	4	Many
Duration exposure (days)	70	35	1	30	395	84	56	24	180
kg milk fat/day	0.4	1.4		1.0	0.9	0.9	1.0	0.9	1.2
2,3,7,8-TCDD		35	30	15	30	38	51	35	58
1,2,3,7,8-PeCDD		33	28	10	20	39	27	24	49
1,2,3,4,7,8-HxCDD		17		5.6	8	33	21	15	51
1,2,3,6,7,8-HxCDD	13	14	27	6.4		33	13	18	77
1,2,3,7,8,9-HxCDD		18		3.1		16	10	13	35
1,2,3,4,6,7,8-HpCDD	1.3	3	1.6	0.6	2	3.4	2.0	3.3	18
1,2,3,4,6,7,8,9-OCDD	0.2	4		0.1	0.8	0.7	0.3	0.4	3.7
2,3,7,8-TCDF					2	nd	nd	<0.1	2.8
1,2,3,7,8-PeCDF					4	nd	nd	<0.1	3.8
2,3,4,7,8-PeCDF		25	36	12	50	40	65	17	58
1,2,3,4,7,8-HxCDF			18	4.3	7	24	23	14	33
1,2,3,6,7,8-HxCDF		16		3.6		19	27	15	30
2,3,4,6,7,8-HxCDF		14		4.2		19	20	9	19
1,2,3,7,8,9-HxCDF								<0.1	nd
1,2,3,4,6,7,8-HpCDF		3	1.7	0.4	1	3.4	1.9	3.5	3.1
1,2,3,4,7,8,9-HpCDF		8		0.5		nd	3.6	4.3	4.2
1,2,3,4,6,7,8,9-OCDF		1			1		0.3	0.3	0.4
PCB77				1.2					
PCB126				35					
PCB169				31					

[1] Carry-over rates are estimated from the intake through the feed and excretion in milk at steady state.
[2] Carry-over rates are estimated from the total intake and excretion in milk during depletion.
Source: adapted from Hoogenboom (2005).

much more slowly with average levels of 36 and 30 mg/kg at 90 and 120 days. During this period several other studies were carried out with Arochlor 1254, with reported dose levels varying between 3.5 and 1000 mg/day. Willett *et al*. (1990) evaluated these studies, starting by standardizing the PCB levels determined by gas chromatography and electron capture detection (GC-ECD). When focusing on cows exposed for 60 days or longer, the following relations were obtained between daily dose (in mg) and respectively milk and body fat levels (in mg/kg):

$$[\text{PCB}]_{\text{milk fat}} = 0.28 \times (\text{daily dose})^{0.82}$$

$$[\text{PCB}]_{\text{body fat}} = 0.16 \times (\text{daily dose})^{0.85}$$

McLachlan *et al.* (1990) and McLachlan (1993) investigated the transfer of dioxins, PCBs and organochlorine pesticides at normal background levels from feed to milk of one lactating cow. Contaminants were analysed in milk, urine and feces. Daily exposure to contaminants came primarily from the feed and varied between 30 and 7600 ng for PCBs. It was shown that in general the transfer to milk increased with higher chlorination of the PCBs, in particular when a congener is substituted at the 4 and 4′ positions. Under these steady-state conditions, the carry-over rates from feed to milk varied between 12% for the labile congeners (like PCB 28) to more than 78% for the most persistent congeners, including the indicator PCBs 118, 138, 153 and 180. In this study the carry-over rate (COR) was defined as the ratio of the amount excreted in milk and the total amount excreted in milk and feces. The pesticides HCB and p,p′-DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene) showed similar high CORs.

Similar carry-over rates were obtained by Thomas *et al*. (1999a, b), who performed a three-month carry-over study with five lactating cows. Animals were exposed at background levels (54 μg total PCB per day; 53 congeners analysed) using 'naturally incorporated' feedstuffs (Fig. 8.3). The seven indicator PCBs contributed 10% to this intake. In this study total daily PCB excretion in milk tended to decrease with increasing lactation period, partly due to decreasing milk gift and partly due to decreasing levels. No difference was found in PCB levels between morning and afternoon milkings. Average PCB levels in milk were 3.9 μg/kg fat with indicator PCBs (in particular 118, 138, 153 and 180) contributing 79%. Due to the loss of body fat during the lactation period, the input–output balance, i.e. the relative ratio between the intake through the feed and output through the milk, reached levels of 134, 116, 124 and 112% for the more stable PCBs 118, 138, 153 and 180 respectively. PCB levels and congener patterns in the body fat of these animals were very similar within the different tissues and organs and reflected those in the milk. Levels in liver on a fat base were three-fold higher, indicating specific binding of certain PCBs, as is also the case for higher chlorinated dioxins (see below). Total body burdens in these animals were estimated to be 450 μg, with indicator PCBs contributing 73%. The

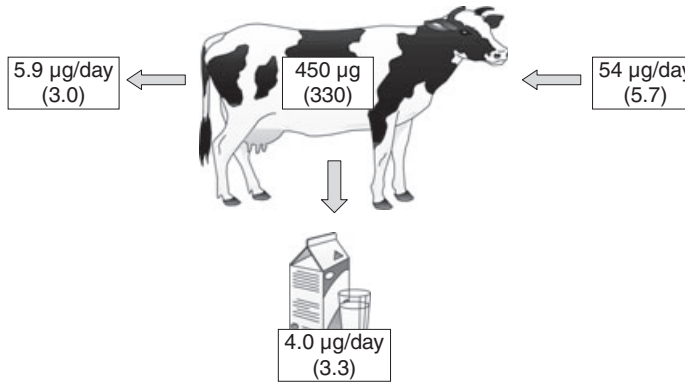

Fig. 8.3 Mass-balance of PCB intake and efflux in lactating cows as determined by Thomas *et al*. (1999a, 1999b). Figures present the sum of 53 congeners and the sum of the seven indicator PCBs between brackets. Level in the cow presents the total amount of PCBs in the animal (body burden), being primarily present in the body fat (Hoogenboom 2005).

95 kg of body fat contained 97.7% of this body burden, and the muscle tissues 1.8% (total body weight 631 kg).

8.4.2 Transfer of dioxins in lactating cows

Many studies have been performed to investigate the transfer of dioxins into milk. Firestone *et al*. (1979) and Jensen and Hummel (1982) exposed lactating cows to respectively pentachlorophenol (PCP) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) contaminated with dioxins. The PCP contained primarily the higher chlorinated 1,2,3,6,7,8-HxCDD, HpCDD and OCDD congeners, the 2,4,5-T only TCDD. Both studies were carried out with relatively high dioxin exposure (respectively 20 and a range of 0.09–9 µg TEQ/day), and therefore might be less representative for general practice. Interestingly, the half-life of 41 days for TCDD, calculated after terminating the 11-week exposure of cows with 2,4,5-T, was similar as determined at lower levels in later studies. Similar is true for the half-lives obtained after the 70-day exposure period for the hexa-, hepta- and octa-CDD, being 76, 88 and 274 days respectively (Firestone *et al*., 1979).

Jones *et al*. (1987, 1989) studied the fate of radiolabelled TCDD in lactating cows given a high single dose of either 30 µg or 45 mg (0.05 and 75 µg/kg body weight) spiked on grain. Seven days after treatment with the high dose, most of the radiolabel was excreted in the feces, with lesser amounts in milk and urine. Fat and liver tissue contained the highest level of radiolabel, being respectively 116 and 76 µg/kg (ww) tissue (levels not corrected for fat content). In the two low-dosed animals, killed after 14 days, levels in fat and liver were more different, being 104 and 8 ng/kg. Blood and milk

levels showed a very similar pattern, with a peak at 1–2 days of respectively 6.5 and 70 ng/kg for cow 1 and 4.5 and 86 ng/kg for cow 2. Respectively 16% and 11% of the radiolabel was excreted in the milk of these two cows. The depletion showed the typical two-phase distribution, i.e. a rapid first elimination, and a much slower second elimination. The activity of ethoxyresorufin-O-deethylase (EROD) and arylhydrocarbon hydroxylase (AHH) in the liver, as measured 7 and 14 days after slaughter, showed a strong and moderate increase in the high- and lowdosed animals. The induction of the cytochrome P450 might be involved in the relatively high-retention of TCDD in the liver, in particular in the case of the high-dosed cow. This may also influence the extrapolation of data from a high dose exposure to more realistic levels that may have no effect on liver enzymes. In the same study two lactating cows received a dose of 0.05 μg/kg radiolabeled TCDD spiked to soil (Jones *et al.*, 1989). Respectively 12% and 14% of the dose was excreted into the milk during the study period of 14 days. Although peak levels in blood and milk appeared to be lower, these data do not suggest a big difference in the uptake of TCDD added to either grain or soil.

Later studies were performed under more realistic conditions with long-term exposures. Under steady state and at background exposure levels, McLachlan *et al.* (1990) found carry-over rates of 25–35% for the most toxic congeners, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF, 15% for the hexachlorinated congeners and less than 5% for the hepta- and octachlorinated congeners (Table 8.2). Cows were exposed to 7.5 ng TEQ/day and excreted 1.6 ng TEQ/day (21%) into the milk and 4.2 ng TEQ/d (56%) in the feces. The remaining 23% was not accounted for, in particular due to the loss of TCDF, 1,2,3,7,8-PeCDF and HpCDD, which are most likely metabolized. Lower-chlorinated congeners were better absorbed than higher-chlorinated congeners, an observation confirmed by other studies. Similar congener-specific effects were observed by Fürst *et al.* (1993) who investigated the relation between levels in soil and milk, and levels in grass and milk at a number of sites. Their data clearly show that at that stage grass but not soil levels were important for milk levels. Similar was concluded by Fries (1987) and Derks *et al.* (1994), the latter based on the decrease of dioxin levels in milk and grass, but not soil near waste incinerators. Of course this situation may change when deposition on grass decreases whereas soil levels remain rather constant.

Also in the Netherlands, one of the first incidents with dioxins was the presence of these compounds in milk from cows grazing in the vicinity of municipal waste incinerators (MWI) (Olie *et al.*, 1977; Liem *et al.*, 1991). Levels exceeded 6 ng TEQ/kg, the Dutch limit at that time. The area was closed for dairy cows, the MWIs were adapted with filters and the cows were transferred to a rural area. Several studies were carried out to investigate the carry-over to milk and in particular the depletion kinetics. Olling *et al.* (1991) exposed four cows to a high single dose of eight congeners each

at 3.7 or 37 ng/kg body weight dissolved in oil. Half-lives in milk fat varied from 27 to 34 days for the heptachlorinated congeners, and from 40 to 49 days for the tetra-, penta- and hexachlorinated PCDD/Fs. TCDF showed a half-life of 1 day and was not detected in the body fat, again demonstrating that this compound is actively metabolized in cows. Levels in milk and body fat were in the same order of magnitude, with the exception of the liver showing relatively high levels. The latter may be explained by the binding of, in particular, the higher-chlorinated congeners to specific proteins.

In a follow-up study Slob *et al*. (1995) treated lactating cows with a single dose of contaminated fly ash (14 μg TEQ) and obtained very low carry-over rates, being 0.35 and 0.53% for the total TEQ level. This study also included a field study with cows on a farm in the vicinity of a waste incinerator. Levels in grass were around 10 ng TEQ/kg dw, and levels in milk around 10 ng TEQ/kg fat. Carry-over rates were calculated to vary between 15% for TCDD and less than 1% for the hepta- and octachlorinated congeners, TCDF and 1,2,3,7,8-PeCDF (Table 8.2). The carry-over rate for the total TEQ was only 7.5%, being three-fold lower than the rate reported by McLachlan *et al*. (1990). This study was the only one that included carry-over of the dioxin-like non-ortho PCBs 77, 126 and 169, showing carry-over rates of respectively 1.2, 35 and 31%. These data show that the most important dioxin-like PCB 126 has a relatively high carry-over rate even when compared to TCDD. Another carry-over study with four cows was carried out, in order to investigate the depletion kinetics of the different dioxin congeners (Tuinstra *et al*., 1992). Cows were fed with contaminated grass and an additional amount of dioxins spiked to briquettes. This resulted in dioxin levels in milk around 50 ng TEQ/kg fat. Cows were subsequently fed clean grass. As in previous studies, it was shown that the elimination half-lives for the three most important dioxin congeners in these cows varied between 63 and 76 days. Schuler *et al*. (1997a) performed a field study near a modern waste incinerator and calculated transfer rates both from air to grass and from grass to milk. Total TEQ in grass was around 0.6 ng TEQ/kg dw and daily intake of dioxins about 10 ng TEQ, leading to concentrations in milk of 1–3 ng TEQ/kg fat. Carry-over rates were similar to those described by other authors, and were about 10–20% for total TEQ (Table 8.2).

McLachlan and Richter (1998) repeated their earlier study, this time with four lactating cows. After 12 weeks of exposure at background levels, cows were fed with grass obtained from a field that had received repeated applications with sewage sludge for 24 days. In particular the intake of hexa-, hepta- and octachlorinated PCDDs and hepta- and octachlorinated PCDFs increased dramatically. Despite the low TEF values of these congeners, total TEQ increased from about 1.5 to 12 ng TEQ/day. As before, carry-over rates to milk decreased with increasing chlorination from about 40% for TCDD and PeCDD to 0.5% for OCDD during the first phase (Table 8.2). In phase 2, carry-over rates for those congeners where the intake clearly

increased were a factor of 2 lower. This can be explained by the fact that the grass was fed for only 17 days prior to sampling. As a result most of the compounds are likely to be accumulated in the body fat and liver, and a steady state is not yet obtained. In this study the levels in the different tissues were very similar when based on fat. Low levels in feces and absence from milk again indicate that TCDF and 1,2,3,7,8-PeCDF are metabolized in cows. In general the fraction excreted in the feces increased with higher-chlorination grade, indicating a lower absorption of these more lipophilic compounds. The authors conclude that the sewage sludge has no effect on the absorption and carry-over to milk. Fries *et al*. (1999) studied the fate of the higher-chlorinated dioxins by feeding dairy cows with PCP-contaminated wood. Four Holstein cows received a daily dose of 70 ng TEQ for 58 days. Steady state was obtained at the first sampling point at 30 days with milk levels of 6 ng TEQ/kg fat. Carry-over rates for individual congeners are in the range reported by other authors (Table 8.2). About 9% of the total ingested TEQ level was excreted into the milk. This rather low figure can be explained by the high chlorination of the congeners, with HpCDD contributing almost 50% to the total TEQ intake.

An unusual incident with lactating cows was discovered in 1998 in southwest Germany, where a gradual increase in milk levels was traced back to the use of contaminated citrus pulp from Brazil (Malisch, 2000). The contamination was actually caused by the use of contaminated lime, derived from a PVC production plant. Levels up to 7.9 and 4.3 ng I-TEQ/kg fat were measured in milk and veal fat. In the Netherlands, levels in pooled milk samples during this period increased to a maximum of 5 ng TEQ/kg fat as compared to maximum levels of 2 and 1 pg TEQ/kg by the end of respectively 1999 and 2001 (Baumann *et al*., 2002). Using one batch of contaminated citrus pulp, a carry-over study was carried out with lactating cows. Figure 8.4 shows the levels in milk from two cows that were fed with the contaminated citrus pulp daily for a period of four weeks (Traag *et al*., 1999). The pulp was contaminated with 10 ng TEQ dioxins/kg, which is about 15 times the current limit of 0.75 ng TEQ/kg. It is evident that this exposure rapidly led to increased dioxin levels in milk, but also the characteristic rapid decrease in the first period after termination of the exposure. The depletion showed the typical two-phase disposition profile and the half-life of elimination was calculated to be 20 days. This half-life was shorter than the half-life calculated by Malisch (2000) for cows on a farm that had received contaminated pulp for six months, being 8–10 weeks for the total TEQ level and the levels of the two most important congeners 2,3,7,8-TCDD and 1,2,3,7,8-PeCDD. A possible explanation is a much more extensive distribution and higher storage in lower perfused fat tissues at prolonged exposure. Carry-over rates were also estimated and are shown in Table 8.2. The carry-over rate for the total WHO-TEQ was estimated to be 44%. During another incident in 2004, with potato peels mixed with contaminated kaolinic clay, an estimated intake of 90 ng TEQ per day resulted in

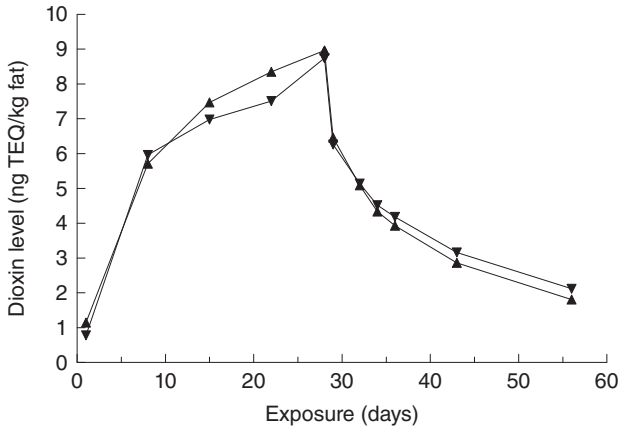

Fig. 8.4 Dioxin levels in milk from two cows fed 5.4 kg of citrus pulp per day for four weeks at a level of 10 ng TEQ/kg. Subsequently, cows received clean feed for another four weeks (Traag *et al.*, 1999).

a steady-state level in milk around 23 pg TEQ/g fat (Hoogenboom *et al.*, 2010). This would amount to a carry-over rate of 26% for dioxins in the clay. TCDD and PeCDD were the most important congeners in the milk. In the clay also the HxCDDs contributed to the TEQ level but in milk they were less relevant, again showing the higher CORs for the lower chlorinated congeners.

8.4.3 Fate of dioxins in beef animals

The first study on the fate of dioxins in beef cattle was carried out by Jensen *et al.* (1981), who treated calves with feed mixed with 2,4,5-T contaminated with 0.06 mg/kg TCDD. Seven animals of about 200 kg received a daily dose of 0.15 μg TCDD per day for 28 days, amounting to a total dose of 4.2 μg. At that time three animals were slaughtered; the others were followed for depletion kinetics and slaughtered after 50 weeks. Levels of TCDD in fat, liver and muscle, just after the exposure period, were 84, 9 and 2 ng/kg tissue respectively. Correction for the fat content in muscle (2%) showed that the levels on a fat base were similar in fat and muscle tissue, leading to the conclusion that fat is the most suitable tissue for monitoring for TCDD exposure. Using a 3–4% fat content in liver (Feil *et al.*, 2000), TCDD levels in liver fat would be 200–300 ng/kg, indicating selective retention in liver. During the elimination period, the half-life for TCDD was calculated to be 16.5 weeks, based on levels in fat biopsy samples. It should be noted that during this 50-week withdrawal period, animal weights increased from 210 to 313 kg. This implies that at least part of the decrease in the levels is due to a dilution by the increase of body weight and, more

important, body fat. It is unclear whether the high dose of 2.5 g/day 2,4,5-T has any influence on the kinetic behaviour of TCDD.

In order to determine bioconcentration factors (BCFs) in beef animals, Feil *et al*. (2000) fed four growing calves with feed enriched with a number of selected dioxins and dioxin-like PCBs for 120 days. The animals received a daily dose of 225 ng TEQ (83–750 ng/congener). During this period the weight of the animals increased from 250 to 350 kg and the percentage of body fat from 4 to 10%. In addition four control animals were included and analysis of tissues of these animals showed that a second contamination source was present. This source was shown to be wood posts treated with PCP. As a result both the control and dosed animals showed high levels (100–1500 ng/kg fat) of hexa-, hepta- and octachlorinated dioxins, which are typical contaminants in PCP. Both the hepta and octa congeners accumulated in the liver. The tetra- and pentachlorinated dioxins and furans were only observed in the dosed animals and reached levels in back and perirenal fat of up to 300 (TCDD), 100 (PeCDD), 5 (TCDF) and 100 (2,3,4,7,8-PeCDF) ng/kg. Fat-based levels of TCDD in ribeye were twice as high. TEQ levels reached maximum levels of 260 and 20 ng TEQ/kg fat in back fat of dosed and control animals. Based on these data, about 30–50% of TCDD, PeCDD and PeCDF was retained. As in other animals TCDF was only retained for 1%. A follow-up study on PCP-contaminated wood in animal facilities showed that this source may still be responsible for a large part of the increased residues in beef from US cattle (Fries *et al*., 2002a). A close relationship was observed between dioxin and PCP levels in wood, showing that the latter may serve as a good indicator for contamination.

Thorpe *et al*. (2001) treated 10 Holstein-Friesian heifers with five lower chlorinated dioxin congeners for four weeks at a dose of 150 ng/congener/day or 405 ng TEQ/day. This was followed by withdrawal periods of 1, 14 and 27 weeks. Initial levels in fat varied from 48 ng/kg for TCDD to 22 ng/kg for 1,2,3,6,7,8-HxCDD but decreased 3–4 fold during the next 26 weeks. During this period animal weights increased from 340 to 490 kg, again showing that part of the decrease in the levels can be attributed to the increase in body fat. Half-lives calculated in fat tissue were calculated to be 93 days for 2,3,7,8-TCDD, 126 days for 1,2,3,7,8-PeCDD, 148 days for 1,2,3,6,7,8-HxCDD, 106 days for 2,3,4,7,8-PeCDF and 124 days for 1,2,3,4,7,8-HxCDF. Levels in muscle fat were initially six times higher than in body fat but decreased to about two times at weeks 14 and 27 (Fig. 8.5). This is in line with the observation of Feil *et al*. (2000). Based on these results, the authors conclude that levels in fat samples taken shortly after exposure may seriously underestimate the levels in other tissues, including meat. A possible explanation is the fact that the perfusion and therefore distribution to the fat is much slower.

In a parallel study of their experiment with lactating cows, Richter and McLachlan (2001) fed four non-lactating cows with grass contaminated at background levels (0.2 ng TEQ/kg) for 11.5 weeks, followed by four weeks

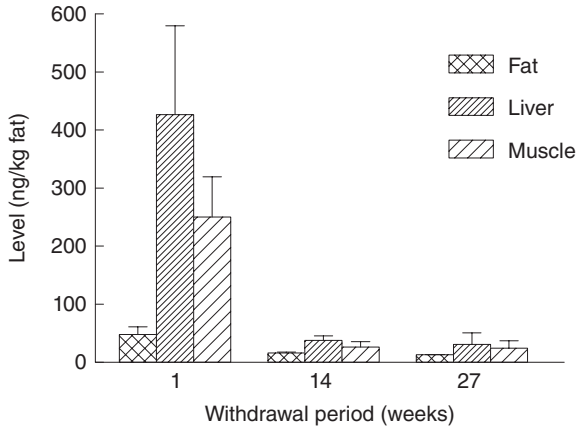

Fig. 8.5 Levels of TCDD in subcutaneous fat, liver and muscle tissue from cows treated for four weeks followed by withdrawal periods of one, 14 and 27 weeks. Similar results were obtained for the other four congeners. Adapted from Thorpe *et al.* (2001) (Hoogenboom, 2005).

on grass contaminated with sludge (1.6 ng TEQ/kg). Two cows each were slaughtered at the end of phases 1 and 2. Average exposure during phases 1 and 2 was respectively 1.4 and 12.5 ng TEQ/day, the increase primarily coming from higher-chlorinated PCDDs. Based on the intake and excretion in feces, there was again the apparent lower absorption with higher-chlorination rate. Very remarkable was the fact that even at the still relatively low levels of exposure during phase 2, the higher chlorinated congeners accumulated in the liver. Based on organ weights it was calculated that at the end of this phase approximately 4, 6, 12, 50 and 73% of the total increase in the body burdens of respectively 1,2,3,7,8-PeCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, HpCDD and OCDD was present in the liver. The authors hypothesized that initially all the dioxins are retained in liver and are subsequently distributed to other tissues. The higher blood/liver partition coefficient for the lower-chlorinated compounds subsequently results in a more rapid distribution of these congeners to the other tissues.

8.4.4 Dioxins and PCBs in chickens

One of the first reported incidents with dioxins in chickens was accompanied by the chicken oedema syndrome, which was reported for the first time in 1957 (Sanger *et al.*, 1958; Schmittle *et al.*, 1958). Millions of chickens became ill or died after eating a feed with toxic fatty acids. These were produced from fat scrapings of cowhides, which were later shown to have been treated with polychlorophenols (reviewed by Firestone, 1973). It took until 1966 to point out dioxins as the causal agent. It was shown that the

toxic fat contained dioxin levels as high as 260 μg TEQ/kg (Higginbotham *et al*., 1968; Hayward *et al*., 1999). In 1969 another outbreak of chicken oedema disease occurred in North Carolina. The cause was traced back to the accidental contamination of vegetable oil with wastewater from a pesticide plant (Firestone, 1973).

In a nationwide survey in 1996 by US-EPA, elevated dioxin levels were found in two out of 80 chicken samples (Ferrario *et al*., 1997; Ferrario and Byrne, 2000). Levels in these two samples were about 37 ng TEQ/kg, which was mainly derived from TCDD (50–60%). The contamination was traced back to the use of contaminated ball clay as an anticaking agent in the feed. Further studies on different batches of ball clay revealed average levels around 1 μg TEQ/kg product, which had been used for less than 1% in feed for chickens, catfish and cows (Ferrario *et al*., 2000). After termination of this use, FDA investigated dioxin levels in catfish and eggs, focusing on the presence of TCDD only as a screening method. In 24 egg samples, all derived from hens exposed to ball clay, TCDD levels varied between 0.6 and 2.5 ng TEQ/kg wet weight. Based on the 60% contribution of TCDD to the total TEQ and 10% fat in the egg, this means levels of about 10–30 ng TEQ/kg fat. Interestingly, the highest levels in this survey were obtained from a producer who used feed without ball clay but who had fortified the feed with fat from contaminated chickens. A similar problem, spreading of the incident through the rendering of contaminated fat, occurred during one of the largest food incidents which happened in January 1999.

In April 1999, RIKILT became involved in the Belgian dioxin crisis after detecting dioxin levels of 780 ng TEQ/kg in chicken feed, 960 ng TEQ/kg in chicken fat and 710 ng TEQ/kg in egg fat (Hoogenboom *et al*., 1999). Guided by the congener pattern, being primarily PCDFs, also PCBs were analysed and shown to be the source, with indicator PCB levels of 31.7, 36.9 and 35.1 mg/kg product in feed, chicken fat and egg fat respectively (Hoogenboom *et al*., 1999). Based on these data, it became apparent that the 80,000 kg of fat used for the production of chicken and pig feed in January had been contaminated by at least 200 kg of PCB oil contaminated with high levels of, in particular, dibenzofurans (PCDFs) (Bernard *et al*., 1999; van Larebeke *et al*., 2001). This amount is in line with the quantity of 50 kg of the seven indicator PCBs described by De Bont *et al*. (2003) knowing that these congeners represent about 30–40% of an Arochlor 1254/1260 technical mixture. Feed and food samples were eventually sent to RIKILT because of problems with the hatching success of eggs, the viability of the young chickens and symptoms in adult chickens resembling the chicken oedema syndrome, pointing in the direction of dioxins. Since the incident was discovered three months after it had occurred, the contamination could spread across the food chain and required the analysis of many food items to prove the absence of dioxins. Since the analysis of dioxins at that time was very difficult and expensive and only a limited capacity was available, it was decided at the end of June to accept PCB analysis as an indicator for

dioxins. Based on a ratio of 50,000 between the sum of the so-called indicator PCBs (PCBs 28, 53, 101, 118. 138, 153 and 180) and dioxins (expressed in TEQs), PCB limits of 200 ng/g fat for animal and egg fat and 100 ng/g fat for milk fat were established in Belgium. It was assumed that the ratio would be constant over time, i.e. kinetics for the indicator PCBs and dioxins in animals were assumed to be identical. Although some studies had been performed in chickens on either dioxins or PCBs, no studies were available to support this assumption.

8.4.5 Transfer of dioxins and PCBs from feed to eggs

In order to study the similarity in kinetics of dioxins and PCBs, various carry-over studies were performed with broilers, laying hens and pigs fed with Belgian feed (Hoogenboom *et al.*, 2004a; Traag *et al.*, 2006). The studies focused on the depletion of dioxins and PCBs following a short exposure period of one week. The mixture of two original chicken feeds, containing 1% and 4% contaminated fat, was diluted 10-fold for this study. Levels of dioxins, non-ortho and mono-ortho PCBs were respectively 61, 23 and 116 ng TEQ/kg. In addition the feed contained 3.2 mg/kg of indicator PCBs, resulting in a ratio between the indicator PCBs and dioxins of 52,000. Figure 8.6 shows the levels of dioxins, non-ortho and mono-ortho PCBs in egg fat. The levels showed a maximum at day 10, i.e. three days after termination of the exposure. This can be explained by the production process of an egg in the ovarium of the hen, which requires about 10 days. Maximum levels of dioxins and total TEQ levels (dioxins and PCBs) were 214 and 675 ng

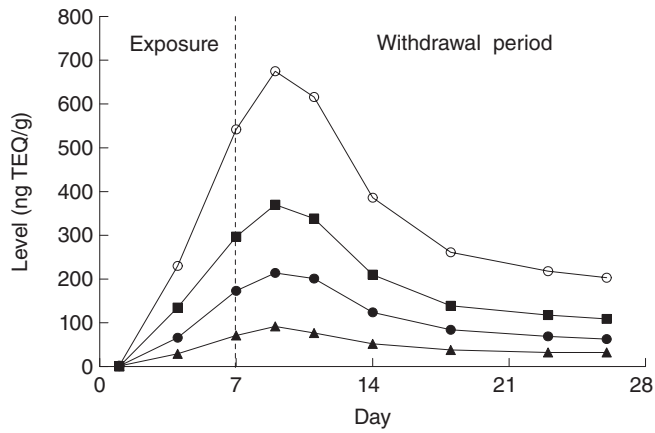

Fig. 8.6 Levels of dioxins (closed circles), non-ortho (closed triangles) and mono-ortho (closed squares) PCBs, as well as total TEQ levels (open circles) in chicken eggs. Chickens were exposed for seven days and subsequently fed clean feed (Traag *et al.*, 2006).

TEQ/kg fat. Based on the 10–15 fold dilution of the original feed, this implies that during the first weeks of the incident total TEQ levels as high as 10 μg TEQ/kg or 60 μg TEQ/egg might have occurred. Such levels were never measured because the first samples of eggs and chickens were collected several weeks after the feed had been replaced by clean feed.

Indicator PCB levels followed a similar pattern as dioxins with a maximum level of 17 mg/kg egg fat at day 10. Lower-chlorinated PCBs 28, 52 and 101 were less present in the fat and more rapidly excreted during the depletion period than PCBs 118, 138, 153 and 180.

Similar results were obtained in a follow-up study with much lower levels of dioxins and PCBs in the feed (Hoogenboom *et al*. 2006b). In this study it was shown that even after 56 days no steady-state conditions were obtained. Following termination of the exposure, levels initially decreased rapidly and subsequently showed a rather slow decrease. This can be explained by the fact that during the first days after termination, most of the dioxins come from the GI tract and highly perfused tissues. However, during the slow phase dioxins are remobilized from the fat storage. The data from this study were used to build a physiological model (van Eijkeren *et al*., 2006). From this model, half-lives for the two phases were calculated to be respectively 2.5 and 50 days. This demonstrates that once the hens produce eggs above the limit, it will take rather long to obtain levels below the limit. Furthermore, this study also showed that the existing EU limit for feed of 0.75 ng TEQ/kg for dioxins should be lowered by a factor of 2–3 in order to guarantee that the levels in the eggs remain below the limit of 3 pg TEQ/g fat. This demonstrates that the laying hen concentrates these contaminants in the eggs and that even low exposure through feed or soil may result in non-compliant levels in eggs. This was actually the case during an incident in the Netherlands in the spring of 2010 when egg levels exceeded the limits 2–3-fold whereas feed levels were around the limit.

8.4.6 Dioxins in eggs from free-range chickens

In 2001 a new monitoring programme was started in the Netherlands, aiming at investigating products of animal origin, like meat, milk and eggs. Samples were screened in the CALUX bioassay and suspected samples investigated by GC/HRMS (Hoogenboom, 2002). In 2001 a total of 57 egg samples was screened. Two of these eggs (3.5%) showed a positive CALUX response and were shown by GC/HRMS to contain dioxin levels of respectively 5.0 and 7.7 ng TEQ/kg fat, and total TEQ levels (including dl-PCBs) of 5.5 and 11.3 ng TEQ/kg. The latter sample was traced back to a small farm with free-ranging chickens, which sold its eggs locally (Traag *et al*., 2002). For comparison, dioxin levels determined in four pool samples prepared from the 57 egg samples varied between 0.5 and 0.7 ng TEQ/kg, and total TEQ levels between 0.8 and 1.5 ng TEQ/kg. This observation indicated that many more farms with free-range chickens might produce eggs with

dioxin levels over the maximum level. Therefore, another 17 farms were selected and visited. Eight of these farms, termed free-range, allowed but did not force their chickens to go outside. As a result, these chickens foraged outside for only a limited part of the day. Nine other farms were so-called organic farms, forcing their chickens to forage outside the stable for at least eight hours per day. Screening of the 17 egg samples revealed six positive samples, all deriving from organic farms. The six positive samples were analysed by GC/HRMS: one sample showed a dioxin level above the Dutch limit at that time of 5 ng TEQ/kg, and two more samples a level at or above the new EU limit of 3 ng TEQ/kg. Total TEQ levels varied between 2.9 and 10.5 ng TEQ/kg, showing that dioxin-like PCBs may contribute significantly to the residue levels.

An attempt was made to find the source of the contamination at the first farm. Feed samples were negative in the CALUX assay, i.e. lower than 0.4 ng TEQ/kg. Furthermore, similar feed was used in farms without free-ranging chickens and as such seemed unlikely to contribute more than 1 ng TEQ/kg fat. Water samples did not contain significant levels. Therefore, it seemed likely that the source of the contamination was outside the stable and possibly related to the ingestion of contaminated soil. Samples of soil and straw collected outside and inside the stable contained dioxin levels of 1.1 and 1.6 ng TEQ/kg and total TEQ levels of 1.3 and 3.0 ng TEQ/kg, which are not abnormal levels in industrialized countries. However, assuming a complete transfer of dioxins to the egg and a fat content of 6 g per egg, a total of at least 60 pg total TEQ should be ingested per day, or at least 46 g of the soil contaminated at 1.3 ng TEQ/kg. Therefore, it cannot be excluded that worms or insects, able to accumulate dioxins, may play a role in the contamination.

Schuler *et al*. (1997b) performed a field survey on chickens from five farms. Soil samples taken from a depth of 0–10 cm were analysed and shown to contain 11 (A), 13 (B), 1.8 (C), 1.3 (D) and 1.4 (E) ng I-TEQ/kg dry weight. Corresponding levels in single eggs were 3.1 and 6.1 (A), 19 and 12 (B), 4.6 and 2.3 (C), 6.1 (D) and 3.5 (E) ng I-TEQ/kg fat. The relatively low levels in eggs from farm A were hypothesized to be due to the high density of chickens on this particular farm as compared to the other farms, resulting in the disappearance of soil organisms. Harnly *et al*. (2000) described a more controlled study on a contaminated area close to a pentachlorophenol wood treatment facility in Oroville. Soil concentrations of around 6 ng I-TEQ/kg (range 1.5–46) resulted in egg levels of 20–50 ng I-TEQ/kg fat (range 0.8–140). The fraction of eggs exceeding 10 ng I-TEQ/kg was around 70–90%. Modelling of the data indicated that for confined chickens, a soil level of 2.7 ng TEQ/kg was required to result in an egg level of 10 ng I-TEQ/kg fat. However, a soil level of only 0.4 ng TEQ/kg was required for chickens that had a larger area to forage in. Again, this suggests a role for worms and insects, since their presence is more likely at lower densities.

Wågman *et al*. (2001) showed that worms fed contaminated food are able to accumulate in particular the higher-chlorinated PCBs, mainly due to a slower elimination of these congeners. Matscheko *et al*. (2002) studied levels in soil and worms and observed that the accumulation of certain PCBs from soil to worms is higher than that of 2,3,7,8-substituted dioxins. In general the ratios between worm lipids and soil organic matter were in the order of 1 to 20 for PCBs and 0.1 to 1 for dioxins, with higher accumulation of the tetra- and pentachlorinated PCDD/Fs. Regarding the lipid content of worms (1–2%) and percentage of organic matter in soil (2–6%), levels of PCBs in worms on a wet weight base in general exceeded those in soil. For dioxins this seems to be less the case.

In general these studies confirm that very low soil levels may already result in high levels in eggs, which appear to be a very sensitive product. Additional studies both in the Netherlands and in other European countries confirmed that this issue was more common (Kijlstra *et al*., 2007; van Overmeire *et al*., 2009; Hsu *et al*., 2010; Menotta *et al*., 2010). Stricter control forced the farmers in the Netherlands to improve the situation, e.g. by applying better hygiene (no waste burning, no old equipment and wood, replacement of contaminated soil) at the farms and a better feeding regime to reduce the intake of soil (Kijlstra *et al*., 2007).

8.4.7 Dioxins and PCBs in broilers

Contrary to laying hens, broilers are characterized by a rapid growth and no excretion of dioxins/PCBs through the eggs. As described above, a carry-over study was performed on broilers with the Belgian feed (Hoogenboom *et al*., 2004a). Broilers were fed for one week with the diluted feed described above, followed by periods on clean feed for one or three weeks. Table 8.3 shows the levels of dioxins, dioxin-like (non- and mono-ortho)

Table 8.3 Levels of dioxins, non-ortho and mono-PCBs, and indicator PCBs in fat of broilers fed with contaminated feed for one week followed by clean feed for one or three weeks. Fat samples from five broilers per sampling point were pooled[1]

Week after treatment	Dioxins (ng TEQ/kg)	Non-ortho PCBs	Mono-ortho PCBs	Total	Indicator PCBs (µg/kg)
0	102	84	216	402	6234
	(100)	(100)	(100)	(100)	(100)
1	55	41	121	217	3165
	(54)	(49)	(56)	(54)	(51)
3	26	22	61	109	1522
	(25)	(26)	(28)	(27)	(24)

[1] Figures between parentheses indicate the percentage compared to week 0.
Source: Hoogenboom *et al*. (2004a).

PCBs and indicator PCBs. The major decrease in tissue levels appears to be due to the growth of the animals. Based on normal growth curves, body weight will increase from 800 g at week 0 to respectively 1500 and 2500 g at weeks 1 and 3. Fat levels will increase from 13 to 14 and 16%, resulting in body fat levels of about 100, 200 and 400 g. As can be seen from Table 8.3, dioxin and PCB levels showed a similar two- and four-fold reduction, thus suggesting very little metabolism and excretion. No congener-specific accumulation and depletion was observed, with the exception of levels of PCB 77 which were relatively low at week 0 and decreased more than six-fold during the depletion phase.

De Vos *et al*. (2003) performed a carry-over study with the seven indicator PCBs at levels as low as 3 and 12 μg/kg feed, being below the Belgian limit of 200 μg/kg fat. Broiler chickens were exposed during their six week life time. Fat levels in feed were also varied, being 4, 6 or 8%. During the last 29–42 days the intake and output through feces was monitored, resulting in bioavailability rates of 68 and 82% for the 3 and 12 μg/kg feeds. Levels in abdominal fat, thighs and breast were respectively 26, 20 and 63 μg/kg fat at the lower PCB level, and 117, 41 and 107 μg/kg fat at the high level. The percentage of fat in the feed did not influence the bioavailability and levels in the tissues. These data suggest that levels in abdominal fat may not be representative for other tissues, but in particular in the breast the percentage of fat is very low, which may easily lead to variations in the figures. No congener-specific data were presented.

8.4.8 Dioxins in pigs

Table 8.4 shows the levels of dioxins and dioxin-like and indicator PCBs in back fat of three-month-old pigs, fed with 10-fold diluted feed from the Belgian incident for one week, followed by clean feed for up to 12 weeks. Pigs aged three months will normally grow from an initial 20 kg (11% fat) to 110 kg (28% fat) during the experimental period, resulting in an increase in body fat content of 2.2 to 30 kg. The 14-fold increase in body fat reflects the decrease in PCB levels of about 13-fold. Dioxin levels, however, seem to decrease somewhat more rapidly (20-fold). As in the case of cows, both TCDF and 1,2,3,7,8-PeCDF were hardly detectable in the fat, and levels decreased very rapidly during the elimination phase. This was also observed during the Irish incident in 2008 where these two congeners contributed quite extensively in the dried bakery waste but were very low in the pork. Relative levels of other dioxin-like and indicator PCBs initially reflected feed levels, but the lower-chlorinated congeners (e.g. PCBs 101, 118 and 126) were eliminated more quickly than the higher-chlorinated PCBs like 138, 153 and 180. Data from this carry-over study were used to develop a PB-PK model for pigs that was used for risk assessment during the incident with contaminated gelatin fat in 2006 (Hoogenboom *et al*., 2007).

Table 8.4 Levels of dioxins, non-ortho and mono-PCBs (ng TEQ/kg), and the seven indicator PCBs (μg/kg) in fat of pigs fed with contaminated feed for one week followed by clean feed for 15 weeks. Biopsy samples were obtained from five pigs and pooled prior to analysis. Levels of the seven indicator PCBs were determined in fat from individual pigs (n = 5 per sampling point)[1]

Week	Dioxins (ng TEQ/kg)	Non-ortho PCBs	Mono-ortho PCBs	Total	Indicator PCBs (μg/kg)
0	26.1 (100)	15.3 (100)	81.9 (100)	123.3 (100)	3614 (100)
1	21.8 (83)	10.3 (67)	63.0 (77)	95.1 (77)	2566 (71)
2	15.0 (57)	6.4 (42)	48.1 (59)	69.5 (56)	1787 (49)
4	7.4 (28)	3.0 (20)	29.4 (36)	39.8 (32)	1244 (34)
8	3.3 (13)	1.3 (8)	17.7 (22)	22.3 (18)	866 (24)
12	1.3 (5)	0.6 (4)	10.3 (13)	11.9 (10)	603 (17)

[1] Figures between parentheses indicate the percentage compared to week 0.
Source: Hoogenboom *et al*. (2004a).

8.5 Brominated flame retardants

Flame retardants are compounds used to decrease the flammability of plastics and thereby the risk of fire. Several classes of compounds have been or are being used, like the polybrominated biphenyls (PBBs), the polybrominated diphenylethers (PBDEs), tetrabromobisphenol-A (TBBP-A) and hexabromocyclododecane (HBCD) (Fig 8.7). PBBs are no longer produced and have been replaced by other compounds. PBDEs have been produced in the Netherlands, France, Great Britain, the United States and Japan, primarily as the tetra-, penta-, octa- and deca-mixtures (Law *et al*., 2006). The penta-mixture contains primarily tetra- and penta-BDEs, the octa-mixture hepta- and octa-BDEs and the decamixture deca-PBE. High quantities are added to plastics like polyurethane foam. TBBP-A is used as an additive in electronic equipment (circuit boards). Use of the tetra- and penta-PBDE mixtures has been prohibited in Europe since 2003 but the deca-mixture is still used.

PBBs were involved in one of the most tragic accidents in food-producing animals, the Michigan incident, which occurred in the autumn of 1973 (Carter, 1976). The feed additive Nutrimaster (magnesium oxide) was accidentally substituted with FireMaster FF-1, a flame retardant based on polybrominated biphenyls (PBBs). Total PBB intakes as high as 250 g per cow in about 16 days were estimated, initially resulting in symptoms such as body-weight

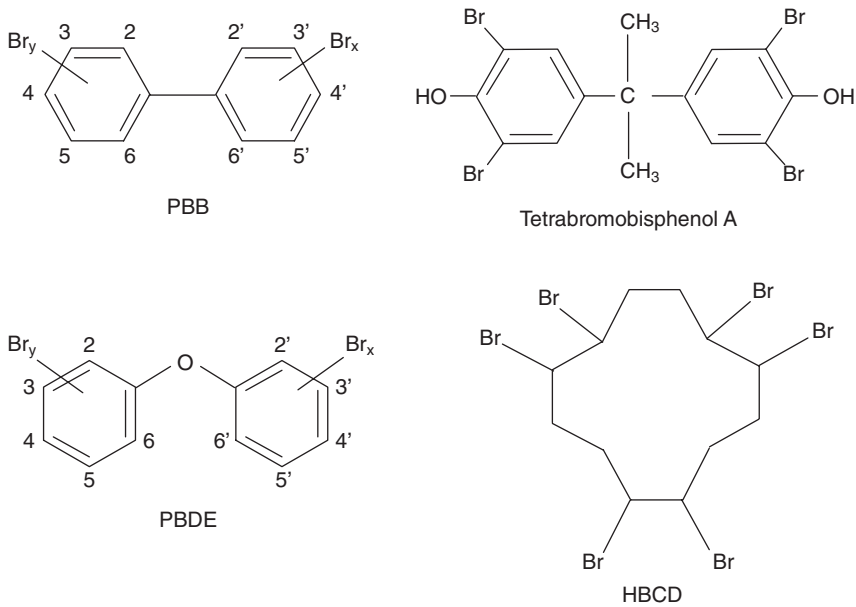

Fig. 8.7 Structures of the most important polybrominated flame retardants. In the case of the PBDEs, congeners numbered 47, 99, 100, 153, 154 and 209 correspond to respectively 2,4,2′,4′-tetraBDE, 2,4,5,2′,4′-pentaBDE, 2,4,6,2′,4′-pentaBDE, 2,4,5,2′,4′,5-hexaBDE, 2,4,5,2′,4′,6′-hexaBDE and 2,3,4,5,6,2′,3′,4′,5′,6′-decaBDE.

loss, decreased milk production and a very typical effect on hoof growth. Fries (1985) estimated that up to 250 kg of FireMaster was fed to livestock, of which 125 kg was eliminated through the feces and 94 kg ended up in human foods before regulation. Elevated levels of PBBs can still be measured in the milk of women in the affected area. In general, the use of PBBs has stopped and these compounds do not appear to be a major concern any more. However, this may not be the case for the PBDEs and HBCD.

8.5.1 Toxicity of flame retardants

In general the data on the toxicity of the PBDEs is still limited (Darnerud *et al*., 2001). The decabrominated compound has been tested extensively, showing that the compound is not mutagenic or genotoxic (Hardy, 2002). There are, however, indications for weak carcinogenic effects based on tumours in livers of male mice. Preliminary reports on the potential binding of the non-planar compounds to the Ah receptor (Meerts *et al*., 1998; Brown *et al*., 2004) have now been attributed to impurities. This is shown by more recent data from studies with purified standards (Peters *et al*., 2006). More recent data show that PBDEs are able to activate the so-called CAR (constitutive active receptor) and PXR (pregnane X) pathways. Activation of

these pathways leads to induction of various biotransformation enzymes including CYPs 2B1 and 3A1/3, resulting in disruption of hormone homeostasis such as from thyroid and sex steroid hormones. This may be the mechanism underlying the observed effects on neurodevelopment, behaviour and possibly also reproduction.

In the case of PBDEs, there are indications for an increased sensitivity of the fetus. As in the case of non-dioxin-like PCBs, exposure of young animals to the pentabromo congener BDE-99 (0.8 and 12 mg/kg bw/day) resulted in decreased learning and behavioural disturbance at a later age (Eriksson *et al*., 2001). Comparable effects, but to a lesser extent, were observed for the tetrabromo congener BDE-47, whereas TBBP-A showed no effects. Effects on behaviour have also been reported for the decabromo congener BDE-209 (Viberg *et al*., 2003, 2006; Johansson *et al*., 2008). Similar to certain non-dioxin-like PCBs, PBDEs can affect the homeostasis of thyroid hormones. The no-adverse-effect level (NOAEL) for this effect of the pentabromo-BDE is around 1 mg/kg bw/day. Using a factor of 10, a NOAEL of 0.1 mg/kg bw was calculated by Darnerud *et al*. (2001). However, as in the case of dioxins and PCBs, in order to extrapolate the NOAEL from animal studies to humans, it is essential to take into account potential species differences in the kinetics and accumulation. Bakker *et al*. (2008) used the very limited data about kinetics and calculated a human NOAEL for BDE-99 of 0.2–0.3 ng/kg bw/day. This limit was based on the effects of BDE-99 on reduced sperm counts in rats, following exposure of the dams (Kuriyama *et al*., 2005) with a lowest observed adverse effect level (LOAEL) of 60 μg/kg bw. An uncertainty factor of 95 was used to extrapolate to a NOAEL and to account for inter- and intraspecies differences.

EFSA (2011) recently evaluated the potential risks of PBDEs and concluded that for BDEs 47, 99, 153 and 209 respectively sufficient data were available to estimate a margin of exposure (MOE) but not to derive an exposure limit. The neurodevelopmental effects were considered to be the critical endpoint. A so-called benchmark dose for 10% of the effect ($BMDL_{10}$) was estimated to be 309, 12, 83 and 1700 μg/kg bw for BDEs 47, 99, 153 and 209 respectively. To account for differences in the kinetics between rats and humans, this dose was subsequently translated to a critical body burden and the daily exposure leading to such levels in humans. Based on the current exposure of the European population, MOEs for the average exposure were 90, 6.5, 23 and 97 000 for BDEs 47, 99, 153 and 209 respectively. For the highly exposed consumers these MOEs were smaller, being respectively 38, 3.9, 14 and 97,000. Due to their relatively higher consumption, young children showed lower MOEs and in the case of BDE 99 these were 1.4 and 0.7 for average and high consumption respectively. An MOE larger than 2.5 was considered to indicate no health concern. This low value was based on the fact that differences in kinetics and also differences in susceptibility between humans and rats were already covered by the approach. This shows that for BDE 99, the exposure was the most critical

and within a range causing some concern. For BDEs 47, 153 and 209 the current exposure is of no concern. However, the EFSA evaluation shows that further studies are required to deal with the various uncertainties, including the additive effects of the various BDEs.

The toxicity of HBCD has been investigated by the producers and is summarized in an EPA report (ACC, 2001; Hardy, 2001). HBCD consists of three stereo isomers (α, β, γ) which contribute 6, 8 and 80% respectively. The metabolism in rats is very extensive and more than 80% of a radiolabelled substance was excreted during the first 72 hours. The alpha congener was the relatively most persistent congener in the tissues. This was confirmed in a study with rats by Van der Ven *et al*. (2006). There are no indications that HBCD is mutagenic, genotoxic or carcinogenic. Exposure of rats for 28 or 90 days resulted in reversible effects on liver size, as well as histological effects on liver and thyroid. There was also a decrease in thyroid hormone (T4) levels in the blood. The LOAEL was around 300 mg/kg bw/day, the NOAEL at 100 mg/kg bw/day. Within the EU-FIRE project, the toxicity of HBCD was re-evaluated. A 28-day rat trial was performed with a dose-range varying between 0.3 and 200 mg/kg bw/day. Again, effects on increased liver and thyroid weight, as well as decreased total T4 levels in blood, were observed. Furthermore, the activity of T4-UGT, involved in the glucuronidation of T4, was increased at the higher dose levels. Effects were only clear in female rats. Groups were too small to observe statistical significance for the effects, although a NOAEL of 10 mg/kg bw for the increased thyroid and liver weight seemed obvious from the data. However, using a benchmark dose approach, the authors calculated a BMDL of 1.6 mg/kg bw/day based on the lower 5% confidence interval for a 10% increase of thyroid weight. This BMDL may be regarded as a NOAEL. In this study, this level of exposure resulted in an HBCD level in liver of 40 μg/g lipid. As in the case of PCBs and dioxins, the effects on the thyroid could be caused by the induction of liver enzymes, resulting in an increased degradation of T4 and a feedback regulation on the thyroid. Germer *et al*. (2006) showed that the exposure to HBCD induced drug-metabolizing enzymes probably via the CAR/PXR signalling pathway. The induction of CYPs and co-regulated enzymes of phase II of drug metabolism could affect the homeostasis of endogenous substrates such as steroid and thyroid hormones and as such could underlie the observed effects.

Hamers *et al*. (2006) tested a number of different BFRs and their hydroxylated metabolites in a battery of *in vitro* tests, developed for the detection of hormonal and antihormonal effects. They also included the competition with transthyretin, the transport protein for thyroid hormones. It was concluded that several BFRs have endocrine-disrupting potencies that have not been described before, especially *in vivo*. This may be due to pharmacokinetic aspects but further investigations seem appropriate to exclude these subtle effects.

Recently EFSA (2010) also reviewed the PBBs and it was concluded that the exposure of the European population is of no concern due to the large margin between the current exposure and the NOAEL observed for the most critical effect. In addition it seems unlikely that levels will increase, since PBBs are no longer produced. The critical effect was a hepatic carcinogenic effect observed after treatment of rats with a technical mixture (FireMaster FF-1).

The contradictory effects of PBDEs on the Ah-receptor may be explained by the presence of brominated dioxins in the test compounds, but at the same time there are strong indications that such impurities are also present in the technical products used in practice. Furthermore, they may be formed during combustion of PBDEs (Sakai *et al*., 2001). Although data on the toxicity of brominated dioxins are rather limited, Birnbaum *et al*. (2003) concluded that they are similarly toxic as their chlorinated counterparts. Based on the criteria described above, including their mechanism of action, they should be included in the TEQ principle and may in practice be added to the dioxins and dioxin-like PCBs present in food. Whether these compounds would actually contribute to a significant extent to the TEQ levels in, for example, food is unclear, since very limited data are available on their occurrence in food. More data are available on the PBDEs and several recent studies have estimated the current exposure to these compounds (see below).

8.5.2 Analysis of flame retardants

Most of the brominated flame retardants like the PBBs and PBDEs can be analysed by GC using ECD or MS detection. In particular, BDE 209 may cause problems and requires specific measures. HBCD, being a mixture of three different congeners, can be analysed by LC/MS. Brominated dioxins, like their chlorinated counterparts, require GC/HRMS analysis. However, these compounds are also detected in the CALUX screening assay (Brown *et al*., 2004). Recently, Traag *et al*. (2009) reported the presence of various brominated flame retardants, as well as brominated dioxins, in the feed additive choline chloride. The contamination was discovered by following up on false-positive results in the CALUX bioassay. Fortunately the levels were too low to cause direct concern, but the discovery points to the fact that production lines of these products are not separated, possibly resulting in a much larger incident in the future.

8.5.3 Exposure to flame retardents

Darnerud *et al*. (2001) estimated the average exposure in Sweden at 51 ng/day based on market basket samples. This study was based on the congeners

47, 99, 100, 153 and 154. Using the same congeners, Ryan and Patry (2001), Lind *et al*. (2002) and Wijesekera *et al*. (2002) calculated intakes for the Canadian, Swedish and British population of respectively 44, 41 and 91 ng/day. Bocio *et al*. (2003) calculated from the total diet study mentioned above for dioxins (Llobet *et al*., 2003) a slightly higher intake for the Catalonian population, of around 90 ng/day. An even higher intake was calculated by de Winter-Sorkina *et al*. (2003) for the Dutch population, of around 213 ng/day or 3 ng/kg bw/day. However, a more recent study by Bakker *et al*. (2008) calculated a median lifelong exposure of 0.8 ng/kg bw/day. In these studies it was shown that fish, pork, dairy and beef contributed most to the intake. Schecter *et al*. (2008) analysed food from the US market and showed that in particular the levels of PBDEs, and as a consequence the exposure, are much higher than in Europe. This is consistent with human milk levels also being an order of magnitude higher in the US.

PBDEs have been detected in human blood and milk in the lower ng/g lipid levels, thus being much lower than the levels of PCBs. Although levels have increased over the last decades, there are some indications that at least for PBDEs these levels have reached their maximum and have started to decline (Sjödin *et al*., 2003). De Winter-Sorkina *et al*. (2003) also estimated the average daily intake of HBCD in the Netherlands and came to a figure of 190 ng/day. This figure was similar to the one estimated by Lind *et al*. (2002) for the average daily intake in Sweden, being around 162 ng/day with a 95th percentile of 332 ng/day. Based on the NOAEL of 1.6–100 mg/kg bw/day mentioned above, it is clear that the margin between the current average exposure around 3 ng/kg bw/day and the toxicological relevant intake is several orders of magnitude. However, this clearly depends on whether the potential difference in kinetics and accumulation is taken into account, as shown by the NOAEL calculated by Bakker *et al*. (2008) for BDE-99 of 0.2–0.3 ng/kg bw/day which would be exceeded by most consumers.

8.5.4 Carry-over of flame retardants in food-producing animals

Carry-over in dairy cows

As mentioned above, one of the most tragic accidents in food-producing animals was the Michigan incident, which occurred in the fall of 1973 (Carter, 1976). The feed additive Nutrimaster (magnesium oxide) was accidentally substituted with FireMaster FF-1, a flame retardant based on polybrominated biphenyls (PBBs). The main constituents of FireMaster are PBB 153, 2,2′,4,4′,5,5-hexabromobiphenyl (47%), and PBB 180, 2,2′,3,4,4′,5,5′-heptabromobiphenyl (24%). As in the case of the Belgian PCB incident, most of the contaminated food was consumed before the problem was discovered. And very similar to that crisis, the cause was only discovered due to the action of individual persons who kept looking for the

agent that caused death and disease of the animals. After elucidating the cause, nine months after the actual incident, Fries *et al*. (1978) were able to obtain 32 cows that had been exposed to 200–400 g PBB over 14 days. Six cows were slaughtered directly and 12 others died or had to be killed within three months. The latter group contained the highest body fat levels, these being 0.2–4 g/kg. Milk fat levels in these cows were 0.1–0.7 g/kg. Fourteen cows were kept on clean feed for 6–9 months and at the end showed body fat and milk fat levels of respectively 20–150 and 16–51 μg/kg. The half-life in milk fat averaged 60 days (range 36–301) (Cook *et al*., 1978). During this depletion phase, there was a good correlation between body and milk fat with the latter being 42% of the former. A small part of the PBBs was excreted through the feces, 10 mg/day, as compared to 65 mg/day via the milk. This shows that there is active elimination of this type of compounds through the feces. Based on the half-life it can be estimated that initial PBB levels in milk were 2–3 g/kg.

Even before this incident, Fries and Marrow (1975) performed a carry-over study with four lactating cows exposed during 60 days to 10 mg/day of the related product Firemaster BP-6 containing the same major congeners, although with a higher hexa to hepta ratio. Cows produced on average 0.6 kg milk fat per day. Maximum levels of the hexa-PBB were 3 μg/g fat and were reached after 20 days. This steady-state condition was reached earlier than in the case of other lipophilic and persistent compounds like PCBs and DDE, the major metabolite of DDT. After termination of the exposure, a two-phase elimination was obtained with a very rapid first phase and a second phase with a half-life of 58 days. Again the decline of 71% during the first 15 days was greater than in the case of PCBs (48%) and DDE (39%). As in the case of PCBs the higher brominated hepta congener was less well transferred to milk.

More recently, Kierkegaard *et al*. (2007, 2009) investigated the carry-over of PBDEs in lactating cows. Samples were derived from the study by Thomas *et al*. (1999a), where two animals were fed with background contaminated silage (levels). The first paper (Kierkegaard *et al*., 2007) focused on the hepta- to deca-BDEs, with BDE 209 being the most important congener in the silage (370–11,000 pg/g dw) and in the feces and lower levels of the nona-BDEs 206, 207 and 208. In the milk this resulted in average levels of BDE 209 of 50 pg/g fat with little variation between the two cows. Similar levels were observed for the octa-BDEs 196 and 197 (35 and 28 pg/g) and the nona-BDE 207 (32 pg/g fat). Overall, the levels of BDEs 206, 208 and 209 in the milk represented less than 1% of the congeners in the feed but for the BDEs 196, 197 and 207 this was up to 41%. The more lipophilic congeners showed a much lower transfer to the milk. However, the balance was hampered by the variability in the BDE-levels in the silage. Six different fat tissues were examined but showed very similar levels when based on a lipid weight. The congener pattern clearly differed from the feed, showing the highest levels for BDE 209 but relatively high levels of the

nona-BDE 207, octa-BDEs 196 and 197 and the hepta-congener 182. The authors suggested that this is unlikely to be due to differences in the absorption or degradation in the rumen but could very well be caused by debromination of the higher brominated BDEs in the body.

The second paper (Kierkegaard *et al*., 2009) focused on the lower brominated BDEs 28, 47, 49, 66, 85, 99, 100, 153 and 154. Highest levels in the silage were observed for BDEs 47 (91–160 pg/g dw) and, 99 (160–230 pg/g dw) which was also the case for the feces. These two congeners were also the dominant congeners in the milk, with average levels in the two cows of 366 and 644 pg/g fat for BDE 47 and 390 and 638 pg/g fat for BDE 99. Levels of BDEs 100 and 153 were about 5–10-fold lower both in the silage and in the milk. On a lipid base, levels in milk and adipose tissue were very similar, contrary to the higher-brominated BDEs from the first study. Contrary to dioxins and PCBs, PBDEs did not accumulate in liver. As a result only 1% of the PBDEs were present in the liver. It was estimated that the absorption efficiency for the tri- and tetra-BDEs was around 80% and for the penta- and hexa-BDEs only 50%. Carry-over rates for the transfer from feed to milk were calculated to be between 15 and 34% for BDEs 47, 99, 100, 153 and 154. This is similar to the rates for PCDD/Fs but lower than for the higher-chlorinated and more persistent PCBs.

Carry-over in laying hens

Fries *et al*. (1976) also studied the carry-over of PBBs to eggs. The study was carried out since chicken feed became contaminated by cross-contamination in mixing plants and maybe also through the rendering of animal material. Ten laying hens were exposed for 63 days with a diet containing 20 mg/kg of Firemaster BP-6, followed by a period of 59 days on clean feed. The hepta congener was excreted in feces to a higher extent than the hexa congener, being respectively 15% and 9% of intake. It was estimated that about 50% and 45% of the hepta and hexa congeners respectively were excreted in the eggs during the first 63 days. Levels of the hexa and hepta congeners in eggs reached a plateau of about 10 and 2 mg/kg egg respectively within 21 days and showed the typical biphasic decrease when the exposure stopped. Levels in body fat of both the hexa and hepta congeners were 40 and 3 mg/kg respectively at day 63, and decreased very slowly during the next 59 days. These data indicate that the PBBs in the body fat are poorly mobilized and transferred to the eggs.

Pirard and De Pauw (2007) carried out a study in laying hens with feed contaminated with PCDD/Fs and BDEs 47, 99, 100, 153, 154 and 183 (tetra to hepta). Dioxin levels were 0.96 ng TEQ/kg and total PBDE levels 3.4 mg/kg. The study was hampered by the fact that laying frequencies were very variable and egg laying stopped almost completely after seven weeks. As a result there was no real steady-state condition. Dioxin levels in eggs steadily increased and exceeded the EU limit of 3 pg TEQ/g within two weeks. Levels reached maximum levels around 10–15 pg TEQ/g fat. This confirms

the results obtained by Hoogenboom *et al*. (2006b). Levels of PBDEs reached a maximum of 22 μg/g fat after five weeks and then decreased to much lower levels at 12 weeks. Levels of PBDEs in liver and abdominal fat were quite similar when expressed on a lipid base, with no clear differences between the relative contribution of the congeners. In the case of dioxins liver levels were slightly higher with a relatively higher-contribution of the higher chlorinated congeners. This study clearly shows that PBDEs are transferred to eggs and tissues of laying hens.

8.6 References and further reading

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8.7 Appendix: abbreviations

2,4,5-T: 2,4,5-trichlorophenoxyacetic acid
AHH: arylhydrocarbon hydroxylase
bw: body weight
EROD: ethoxyresorufin-O-deethylase
GC-ECD: Gas chromatography–Electron capture detection
HBCD: hexabromocyclododecane
HpCDD: heptachlorodibenzo-*p*-dioxin
HpCDF: heptachlorobenzofuran
HxCDD: hexachlorodibenzo-*p*-dioxin
HxCDF: hexachlorobenzofuran
MWI: municipal waste incinerator
OCDD: octachlorodibenzo-*p*-dioxin
OCDF: octachlorobenzofuran
PBB: polybromobiphenyls
PBDEs: polybromodiphenylethers
PCBs: polychlorobiphenyls
PCDD: polychlorinated dibenzo-*p*-dioxins
PCDF: polychlorinated dibenzofurans
PCP: pentachlorophenol

PeCDD: pentachlorodibenzo-*p*-dioxin
PeCDF: pentachlorobenzofuran
SCF: Scientific Committee on Food
TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF: 2,3,7,8-tetrachlorobenzofuran
TEF: Toxic equivalency factor
TEQ: Toxic equivalents
WHO: World Health Organization
ww: wet weight

9

Animal feed contamination by toxic metals

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Abstract: In agricultural regions dietary contamination represents the main source of toxic metals exposure to livestock. This chapter first reviews current data on toxic metal concentrations in animal feedstuffs and husbandry practices related to toxic metal exposure. The chapter then focuses on the effect of toxic metal exposure on animal health and carry-over into food, analysing what are, at the current knowledge, the maximum toxic metal tolerable levels in animal feed that do not pose a risk for animal health and food safety.

Key words: toxic metals animal feed, routes of exposure, animal health, carry-over into food.

9.1 Introduction

Industrial and agricultural development has been largely responsible for pollution of the environment with toxic metals, although some contamination is also derived from natural geological resources.

Most, if not all, elements including essential trace minerals can cause toxic effects to animals and humans if present at inadequate concentrations. With the exception of accidental exposure to very high metal concentrations (such as in polluted areas or from errors in dietary mineral formulations), episodes of essential trace element toxicity are uncommon; this is because organisms have developed important homeostatic mechanisms to limit metal absorption and tissue accumulation. The most important essential trace elements (cobalt, copper, iron, iodine, manganese, selenium and zinc) are routinely added to concentrate feed at levels exceeding 10 times the physiological requirements without any appreciable adverse effect, the most notable exception being chronic copper poisoning in ruminants, especially sheep, due to the extraordinary capacity of this animal species to accumulate copper in the liver.

In terms of potential adverse effects on animal and consequently human health, the heavy metals cadmium, lead and mercury and the metalloid arsenic are amongst the elements that cause most concern. This is because they are readily transferred through food chains and are not known to serve any essential biological function. Although episodes of lethal toxicity associated with accidental exposure to very high doses of toxic metals have been largely described in the literature, the main concern of toxic metals in livestock is dietary exposure, in agricultural regions diet being the main source of toxic metals for animals. Dietary sublethal exposure to these elements can result in adverse effects on a variety of physiological and biochemical processes.

Although contamination of animal feed by toxic metals cannot be entirely avoided given the prevalence of these pollutants in the environment, there is a clear need for such contamination to be minimized, with the aim of reducing both direct effects on animal health and indirect effects on human health.

9.2 Routes of toxic metal contamination of animal feed

When evaluating livestock exposure to toxic metals it is important to consider that dietary exposure will be highly conditioned by husbandry practices, on many occasions minor dietary ingredients and/or supplements, soil ingestion or spurious soil contamination in foliage being responsible for the main toxic metal exposure. For this reason, in addition to toxic metal concentrations in the main feedstuff components, it is important to have in mind how management practices can contribute to enhancing livestock metal exposures in order to minimize their toxic effect on animals, as well as to limit as much as possible metal transfer to human feeds.

9.2.1 Toxic metal concentrations in feed materials

It is difficult to do a comprehensive review of toxic metal content in animal feed materials worldwide, because of limited information concerning the nature of the samples or inadequate sample description (e.g. compound feed without any detailed information on designated species), methods of chemical and statistical analysis (including limits of detection, and values assigned to samples below the quantification limits) and the way data are presented (mean, median, etc.). Tables 9.1–9.4 summarize available data that can be reliably categorized both for feed materials and for commercially manufactured compound or complementary feeds. Most of this information is from official EU organizations, for which individual Member States are obliged to sample and to analyse feed materials as part of the enforcement of feed stuff legislation (see Section 9.7). Little official information exists outside the EU, and although there are some published data

on the scientific literature on toxic metal residues in animal feedstuffs (for a detailed review see Kabata-Pendias and Pendias, 2001, or NRC, 2005) they are generally based on samples collected at a very local scale and/or that are difficult to classify in categories. This information has not been included in Tables 9.1–9.4, even though toxic metal concentrations in feedstuffs from unpolluted areas are in general within the ranges described in the EU data. Most of the available data correspond to compound feed and individual ingredients (e.g. cereals) whereas data on forages are scarce.

Cadmium

Cadmium concentrations in uncontaminated soils range from 0.06 to 1.1 mg/kg DM (Kabata-Pendias and Pendias, 2001), the main factor determining the cadmium content of soil being the chemical composition of the parent rock. Although cadmium uptake by plants is variable depending on soil properties, mainly pH, cadmium concentrations in forages and crops in agricultural regions remain generally below 1.0 mg/kg DM (Table 9.1), even though cadmium concentrations >10 mg/kg DM (10 times higher) were

Table 9.1 Cadmium concentrations (mg/kg DM) in feed materials

	Mean	Median	Maximum	*n*
Single feed components				
Barley grain	0.11	0.08	0.29	6
Citrus pulp meal	0.19	0.10	0.50	10
Fish meal	0.40	0.21	1.4	44
Maize grain and maize by-products	0.06	0.01	0.50	29
Rapeseed, extracted	0.15	0.10	0.50	20
Soya bean meal	0.07	0.03	0.20	17
Sugar beet pulp	0.14	0.09	0.36	12
Sunflower meal	0.41	0.27	1.80	32
Mineral supplements and premixes	0.58		2.34	
Wheat and wheat by-products	0.19	0.13	0.75	27
Forages				
Grass/herbage (fresh)	0.62			1217
Hay	0.73			950
Grass silage	0.09			244
Maize silage	0.28			345
Complete feeds				
Ruminants	0.11	0.11	0.85	358
Poultry – unspecified	0.16	0.16	0.4	33
Poultry – layers	0.16	0.11	0.6	12
Poultry – broilers	0.19	0.16	0.5	8
Pigs < 17 weeks	0.16	0.10	0.50	14
Pigs > 16 weeks	0.07	0.05	0.13	10
Pigs – unspecified	0.09	0.07	0.5	150
Pigs – sows	0.09	0.09	0.16	4

Source: modified from EFSA (2004a).

observed in herbage growing close to industrial areas (Kabata-Pendias and Pendias, 2001). In general, cadmium concentrations in forage crops are higher than in concentrate feed materials; this is associated at least in part, like other toxic metals, with physical soil contamination during harvesting or processing (EFSA, 2004a). Increased cadmium concentrations are regularly found in crops after the application of superphosphate fertilizers; in fact, cadmium-enriched phosphate fertilizers are considered the highest source of cadmium input to agricultural land worldwide (Nicholson *et al*., 1994). Sewage sludge application to agricultural land is also responsible for increases in cadmium concentrations in crops. Mineral supplements and premixes generally contain higher cadmium concentrations than the other main components of the ration (McBride, 1998), though the significance of their cadmium contribution to the total animal intake is difficult to establish because of the low but very variable rate of inclusion.

Lead

Lead concentrations in soils can vary greatly (Kabata-Pendias and Pendias, 2001), and although levels of up to 20–40 mg/kg DM are normal, significantly higher lead concentrations have been reported as a result of industrial and mining activities and application of sewage sludges. Lead uptake by plants is very low and lead residues in forages and agricultural crops are generally below 1.5 mg/kg DM (Table 9.2) and rarely exceed 5 mg/kg DM (Underwood and Suttle, 1999), although lead concentrations in excess of 600 mg/kg DM have been reported in herbage grown on mining soils or to which sewage sludge has been applied. Lead concentrations in forages are generally higher than in concentrate feed materials; however, the low lead uptake by plants indicates that in most cases forages are contaminated with soil or sewage sludges spread on the farmland (EFSA, 2004b). As for the other toxic metals, the processes of harvesting herbage for conservation as hay or silage often result in soil being picked up with the crops, and therefore elevated levels of lead may reflect spurious contamination. Lead concentrations in mineral supplements and premixes can be higher compared to other feedstuffs, though their low proportion in the total diet makes their contribution to the total lead intake low.

Arsenic

Arsenic concentrations in unpolluted agricultural soils range from 0.1 to 40 mg/kg DM (Kabata-Pendias and Pendias, 2001). Arsenic uptake by plants varies greatly, depending on factors such as the amount of soluble arsenic species in the soil, soil properties, redox and pH conditions and microbiological activity, as well as the plant species. Very limited information is available regarding arsenic speciation and metabolism in terrestrial plants, and concentrations are regularly measured as total arsenic. Information on arsenic concentration in single components, apart from in fish and marine products and mineral supplements, is sparse (Table 9.3), although

Table 9.2 Lead concentrations (mg/kg DM) in feed materials

	Mean	Median	Maximum	*n*
Single feed components				
Barley grain	0.97	1	2.19	11
Citrus pulp meal	0.76	1	1.00	14
Fish meal	0.52	0.15	2.22	77
Maize and maize products	0.56	0.31	2.71	31
Rapeseed meal	0.6	1	6.82	18
Soya beans and soybean meal	0.93	1	3.00	21
Sugar beet pulp	1.47	1	4.00	14
Sunflower seeds and by-products	0.37	0.2	1.03	36
Wheat and wheat products	0.26	0.15	0.75	12
Meat meal/meat and bone meal	0.81	0.59	2.00	23
Mineral supplements	3.38	2.56	30.00	198
Premixes	19.05			100
Forages				
Grass/herbage	4.93			1077
Hay	3.89			809
Grass silage	2.02			225
Maize silage	2.19			336
Complete feeds				
Ruminants – unspecified	0.34	0.25	5.3	311
Ruminants – dairy	1.03	1.00	2.0	7
Ruminants – beef/cattle	1.14	1.00	2.0	15
Ruminants – calves	0.82	1.00	1.0	9
Ruminants – sheep	0.64	1.00	1.0	12
Poultry – layers	0.87	1.00	4.9	12
Poultry – broilers	0.52	0.53	1.0	8
Poultry – unspecified	1.16	1.00	4.3	20
Pigs < 17 weeks	0.77	0.64	2.0	13
Pigs > 16 weeks	0.38	1.00	0.5	9
Pigs – unspecified	1.03	1.00	7.2	39
Pigs – sows	0.70	1.00	2.1	5

Source: modified from EFSA (2004b).

generally arsenic concentrations in forages and crops grown on unpolluted agricultural soils remain below 0.5 mg/kg DM (Underwood and Suttle, 1999); arsenic concentrations of up to 73 mg/kg DM were reported in the proximities of industrial smelting plants (Kabata-Pendias and Pendias, 2001). Unlike the main toxic metals cadmium and lead, the oral bioavailability of arsenic from the soil is considerably lower than from water or food, so it can be assumed that soil contamination of forages contributes little to total animal exposure. Within the feed components in animal diets, the highest arsenic residues are found in products of marine origin, such as fish meal and fish oil, even though arsenic is present as relatively non-toxic organic forms (e.g. arsenobetaine and arsenosugars). Arsenic concentrations in mineral supplements are higher than in the main dietary components,

Table 9.3 Total arsenic concentrations (mg/kg DM) in feed materials

	Mean	Median	Maximum	*n*
Single feed components				
Fish meal	4.7	4.21	16.3	95
Fish oil	7.6	8.14	8.9	7
Oil seed meals	0.09	0.04	0.2	17
Maize grain and maize by-products	0.26	0.20	0.51	7
Other cereals and cereal by-products	0.06	0.01	1.08	47
Minerals and mineral supplements (unspecified)	6.8	3.05	15.7	42
Forages				
Grass silage	0.12		0.44	28
Hay	0.05		0.1	2
Maize silage	0.05		0.1	2
Straw	0.05		0.19	4
Complete feeds				
Ruminants – unspecified	0.27	0.26	0.38	4
Ruminants – beef cattle	0.36	0.37	0.60	10
Ruminants – dairy	0.24	0.20	0.49	12
Poultry – layers	0.20	0.25	0.29	3
Poultry – broilers	0.34	0.25	0.60	5
Poultry – unspecified	1.83	3.00	6.70	6
Pigs < 17 weeks	0.72	0.52	2.10	19
Pigs – growers/finishers	0.31	0.06	0.39	4
Pigs – sows	0.85	0.69	5.68	15
Pigs – unspecified	0.62	11.36	5.00	19

Source: modified from EFSA (2005).

though the low inclusion rate makes them a non-significant contribution. Finally, drinking water can be a major source of inorganic arsenic in some geochemical environments (e.g. aquifers under strongly reducing or oxidizing conditions, pH > 8, or mining and geothermal areas; Mandal and Suzuki, 2002) with arsenic concentrations as high as 5 mg/l (in unpolluted fresh waters arsenic ranges between 1 and 10 μg/l).

Mercury

The background mercury concentration in the soil, although not easy to determine due to the widespread mercury pollution, has been estimated as approximately 0.5 mg/kg DM; mercury concentrations exceeding this value should be considered as contamination from anthropogenic sources, mainly coal-burning power plants and other sources of fossil fuel combustion, waste incinerators and crematoria (Nriagu and Pacyna, 1988). In general, mercury uptake by plants is very low and mercury residues are independent of soil mercury concentration, most of the mercury found in foliage having an atmospheric deposition origin (Kabata-Pendias and Pendias, 2001; Ericksen *et al.*, 2003). Mercury concentrations in plants are very low, being

very close to or below the detection limits in most analysed samples; in consequence, only limited information exists in the literature on mercury residues in forages (Table 9.4), it being assumed that mercury exposure in farm animals fed almost entirely on forages in areas not polluted by mercury is very low (ESFA, 2008), anthropogenic mercury exposure accounting for most of the mercury residues in grazing livestock (López-Alonso *et al.*, 2003b). For non-plant feed materials, fishmeal and other fish feeds are the most common sources of mercury for livestock under normal farming conditions, the mean total mercury concentrations being one- to twofold higher than in other feed compounds. Relatively few data are available on the speciation of mercury in fish products; nevertheless the available data

Table 9.4 Total mercury concentrations (mg/kg DM) in feed materials

	Mean	Median	Maximum	*n*
Single feed components				
Barley	0.006	0.001	0.078	29
Wheat	0.003	0.001	0.030	48
Oil seed rape	0.007	0.002	0.100	42
Sunflower meal	0.003	0.001	0.010	13
Soya bean meal	0.022	0.011	0.050	13
Distillers' dried grains	0.047	0.020	0.130	8
Maize gluten feed	0.026	0.015	0.100	15
Vegetable oils	0.021	0.020	0.050	16
Additives and premixtures	0.03	0.01	1.3	290
Minerals and mineral feedingstuff	0.02	0.005	0.59	530
Unspecified feeds and raw materials	0.03	0.01	0.22	238
Complementary feed	0.02	0.01	0.34	228
Calcium carbonate	0.01	0.01	0.03	42
Fish meal	0.10	0.10	0.26	193
Fish and bone meal	0.15	0.15	0.22	13
Fish oil	0.03	0.03	0.21	63
Forages				
Forage crops	0.02	0.002	0.19	368
Alfalfa (lucerne)	0.005		0.02	28
Maize forage	0.007		0.05	42
Complete feeds				
Ruminants	0.012	0.004	0.10	56
Pigs	0.032	0.050	0.050	123
Poultry	0.039	0.050	0.10	96
Horses	0.022	0.010	0.10	9
Mink	0.053	0.054	0.12	39
Rabbits	0.031	0.050	0.10	18
Rodents	0.050	0.050	0.10	25
Dogs and cats	0.02	0.01	0.18	126

Source: modified from EFSA (2008).

(ESFA, 2008) showed that it is mainly present as methylmercury, the most toxic form of mercury. Mercury concentrations in mineral supplements and mineral feedstuffs are low and generally well below 0.1 mg/kg DM. Finally, protein hydrolysates from poultry (chicken and turkey) feathers as feather meal are considered as a potential source of methylmercury contamination in animal feed (Plummer and Bartlett, 1975).

9.2.2 Husbandry practices related to toxic metal exposure

The main point that should be considered when analysing the importance of husbandry practices on toxic metal livestock exposure is extensive versus intensive production systems.

Although it is not possible to make general statements because of the variety of husbandry practices throughout the world, *extensive systems* are represented mainly by ruminants, in which the animals receive a diet based on local products (usually from the animals' own farm), either fresh or preserved, supplemented with concentrate feeds (e.g. cereals, oilseed meals and micronutrients such as minerals and vitamins) to achieve the required level of production (growth rate, milk yield, etc.) which varies through the year, depending on the seasonal feed availability and forage digestibility. The degree of feed supplementation is very variable worldwide, being low in areas with high agricultural land availability but also in sustainable systems. Special mention of the husbandry practices related to toxic metal exposure should be given to organic production systems: these are aimed at reducing environmental contamination, and some practices, such as limiting the use of inorganic fertilizers (e.g. phosphates with high cadmium content) or the use of inorganic mineral supplements in compound feeds, result in toxic metal concentrations in organic feeds being lower than in conventional feeds (Blanco-Penedo *et al*., 2009). From a general point of view, in unpolluted agricultural areas, ingestion of soil when grazing, or when consuming pastures or forages physically contaminated with soil, represents the main source of toxic metal exposure to extensively grown animals. This is because toxic metal concentrations in soils are up to 2–3 times those in plants and it is assumed that ruminants ingest up to 18% of their dietary dry matter when grazing (Thornton and Abrahams, 1983).

A recent study comparing toxic metal accumulation in cattle from different management practices has demonstrated that toxic metal residues are directly related to degree of extensification; animals that obtain most of their feed by grazing have higher tissue toxic metal residues in spite of toxic metal concentrations in feeds being not significantly higher (Blanco-Penedo *et al*., 2009). Although precise toxic metal dietary exposure (as a function of the toxic metal concentration in the feed and the amount of feed consumed) is not easy to calculate in extensive systems, monitoring studies on toxic metal residues in livestock (López-Alonso *et al*., 2002a) indicate that toxic metal concentrations in tissues depend on the geochemical origin of the soil

and could be predicted from it. Even though toxic metal exposure in animals could be very variable between farms located in areas with different soil properties, it will be quite constant within the same farm, subject to changes in management practices, making it easy to monitor toxic metal residues in feedstuffs or animal products in farms located in regions that are contaminated either naturally because of an abnormal toxic metal content in the soils or because of a link to anthropogenic industrial or mining activities.

On the other hand, *intensive systems* are represented mainly by non-ruminant (pigs and poultry) species, whose diet consists almost entirely of concentrate feed, but also highly intensive ruminant systems (feedlots or high-intensive milk production cattle) with a very high rate (up to 90%) of concentrate feed. In these systems, the complete feed given to the animals consists of a range of feed materials from the international or global market selected on the basis of price, availability, and the contribution that they make to the supply of nutrients required by the target animals at a specific level of production. In general, the complete feed is supplemented with certain additives and mineral supplements, which contain, as previously indicated, up to 2–3-fold toxic metal concentrations compared with the main ingredients. However, their contribution to the total metal intake is generally low because of the low rate of inclusion, but can be significant for certain elements such as arsenic and mercury, which are found at very low concentrations in forages and cereals but at very high concentrations in feeds of marine origin. In these systems, toxic metal exposure is very easy to calculate (and as consequence to monitor) as the result of multiplying toxic metal content in the complete feed (as one single analysis) and the amount of ingestion. In addition, and because in these systems diets are highly standardized, intra- and inter-farm variability in toxic metal exposure through the diet is much lower than in extensive systems (Blanco-Penedo *et al*., 2009).

9.3 Animal health risk

It is well established that toxic metals have potential adverse effects in livestock, and naturally occurring episodes of acute and toxic intoxication have been much described in the literature. The clinical forms and the clinical symptoms of toxic metal toxicity have been described (Underwood and Suttle, 1999; NRC, 2005) and are not the interest of this section, which will mainly focus on the maximum tolerable toxic metal level in the diet, defined as the dietary level that, when fed for a defined period of time, will not impair accepted indices of animal health or performance.

Much information exists in the literature on laboratory animals; however, the toxicological database for livestock is very limited in terms of proper dose-response experiments and toxicological endpoints, which makes it impossible to properly establish the concentrations of toxic metals not

associated with risk in animals (NOAELs) or the lowest concentrations associated with toxicity (LOAELs). In addition, some observational studies may have been affected by the presence of confounding factors (i.e. the simultaneous exposure to other metals and/or other pollutants or dietary components) and the time exposure has not always encompassed the full production cycle of the animals.

9.3.1 Cadmium

Manifestation of cadmium toxicity varies considerably depending on dose and time of exposure, species, gender, and environmental and nutritional factors; in addition cadmium tissue accumulation is significantly influenced by dietary interactions with zinc and copper, but also with iron and calcium (López-Alonso *et al*., 2002b). Subsequently, large differences exist between the effects of a single exposure to a high concentration of cadmium or the most common low chronic dietary exposure. In general, clinical signs of cadmium toxicity in animals include kidney and liver damage, anaemia, retarded testicular development and infertility, enlarged joints, osteomalacia, scaly skin, hypertension, reduced growth and increased mortality (Underwood and Suttle, 1999; NRC, 2005).

Minimum toxic doses or maximum tolerable levels cannot be estimated with any precision because of the great variability in cadmium exposure and tissue deposition. Considering a quite constant dietary cadmium exposure during the productive life span, it is generally assumed that for most of the domestic species, cadmium concentrations up to 5 mg/kg DM are most likely to start gross clinical symptoms. Pigs have been considered to be the most sensitive species and clinical signs of toxicity such as growth retardation have been observed at concentrations >4.43 mg/kg DM (King *et al*., 1992). In this species the extensive use of copper and zinc as growth promoters greatly increases the risk of adverse effects, as well as increasing cadmium accumulation in the liver and kidney (Rambeck *et al*., 1991; Rothe *et al*., 1992); these findings seem to be associated with the extraordinary capacity of the pig liver to respond to cadmium exposure with a significant increase in metallothionein synthesis (Henry *et al*., 1994). In ruminants, because of their great susceptibility to copper deficiency, relatively low levels of cadmium in the diet (1 mg/kg DM) have been shown to act as potent antagonists of copper metabolism, leading to secondary copper deficiency (Smith *et al*., 1991; Miranda *et al*., 2005). Cadmium intoxication in other livestock species and in pet dogs and cats is rarely reported.

9.3.2 Lead

Lead is a common cause of accidental poisoning in livestock, episodes of lead toxicity in cattle being relatively frequent because of their natural curiosity, licking and indiscriminate eating habits. Primary sources of lead

exposure to animals include contaminated soils and lead-based products, especially batteries and older paints (Sharpe and Livesey, 2004; Miranda *et al*., 2006). Low levels of lead exposure cause subtle cardiovascular, hematological and neurodevelopmental changes. Higher levels of exposure cause renal, gastrointestinal, hepatic and immunological disturbances (NRC, 2005).

A reliable estimate of dose-dependent effects of lead in livestock is not possible as the relevant information based on experimental studies is lacking. Like the other metals, the toxicity of lead depends on its chemical form, and it is generally recognized that following oral exposure, the toxicity of lead decreases in the following order: lead acetate > chloride > lactate > carbonate > sulfite > sulfate > phosphate. Young animals absorb lead more efficiently than older animals and have a lower tolerance (NRC, 2005).

In pigs, 25 mg/kg DM from lead acetate results in decreased growth, but there is insufficient information to establish a maximum tolerable dose in this species. In ruminants, 250 mg/kg lead in the diet can be tolerated for several months without significant effects on performance; however, levels of lead in kidneys and bone become of concern if consumed by humans. Dogs tolerate 10 mg lead/kg DM without changes in functional indices in hematopoiesis or kidney function. In chickens slight but significant changes in growth and egg production occur with the addition of 1 mg Pb/kg DM as lead acetate, and 0.5 mg/kg of highly soluble lead source appears to be the maximum tolerable dose for chronic exposure in these species when dietary calcium levels are low; however, when dietary calcium levels are high, 100 mg Pb/kg DM is tolerated. It should be noted that the above maximum tolerable levels (NRC, 2005) are for highly available sources such as lead acetate, so animals are likely to be able to tolerate higher levels of many other lead sources. Based on these toxicological data, the lead concentrations found in commercial feed are generally too low to induce clinical signs of toxicity. However, individual intoxications may result from ingestion of feed material originating from polluted areas; in fact, incidental poisonings related to waste disposal are regularly reported. It is therefore recommended to monitor areas where sewage has been applied, but also farmland in close proximity to industrial activities likely to emit lead.

9.3.3 Arsenic

Arsenic generally is not accepted as an essential element, but studies with goats, chicks, hamsters and rats suggest that it may have an essential or a beneficial function in ultra-trace amounts (micrograms per kg diet). At high concentrations all livestock species are susceptible to the toxic effects of inorganic arsenic. Signs of chronic arsenic intoxication include depressed growth, feed intake, feed efficiency, and, for some species, convulsions, uncoordinated gait and decreased haemoglobin (NRC, 2005).

The highest dietary level at which arsenic has no adverse effect, as well as the lowest level that induces toxicosis, largely varies with animal species and arsenic compounds. NRC (2005) has suggested a maximum tolerable arsenic level for domestic animals of 30 mg/kg DM. The toxic dietary concentrations of arsenic are generally 2–3-fold greater than the concentrations normally found in animal feeds. In addition, marine organisms, which have been identified as the major sources of feed contamination with arsenic, accumulate the metalloid predominantly as non-toxic arsenobetaine and arsenocholine. Thus, except in localized areas where arsenic levels are extremely high in drinking water, or major arsenic contamination by mining and smelting industries has occurred, the available data suggest that the non-toxic organic arsenic in feed materials does not pose a significant health risk to animals.

9.3.4 Mercury

While there is a large amount of data on mercury dose–response effects in laboratory animals, few and rather old data are available for livestock, mostly focused on clinical signs of toxicity observed in acute situations. Because of their differing bioavailabilities and tissue distributions, the toxicity profiles of organic mercury and inorganic mercury differ: accumulation of inorganic mercury in the kidneys causes changes in renal function, whereas the easy transport of methylmercury into the brain and across the placenta makes the nervous system and the fetus sensitive indicators for the organic form (NRC, 2005).

The health risk to livestock from dietary mercury exposure is difficult to evaluate because most information on mercury residues in feedstuffs is given as total mercury concentrations and the methylmercury is the form of greatest toxicological concern. However, taking into account that the main sources of mercury are marine products in which the predominant form is methylmercury, from a conservative position it could be assumed that all mercury in feedstuffs is methylmercury. The EFSA has recently reviewed the risk of potential adverse effects of dietary mercury at concentrations allowed in the EU (EFSA, 2008). Among domestic animals, cats are the most sensitive species to methylmercury toxicity; based on the scientific literature a NOAEL of 0.33 mg/kg feed was identified, which indicates that the current regulatory maximum limit for pets (0.4 mg/kg feed; see Table 9.5) is not protective enough. However, based on the available data on the occurrence of total mercury in complete feedstuffs, it is unlikely that these species will be exposed to toxic levels from feed, unless fed almost exclusively home-made diets based on raw fish. Fur animals also seem to be quite sensitive to mercury, and although mink will be able to tolerate the maximum levels set for total mercury in complete animal feedstuffs in the EU, it cannot be excluded that the extensive use of offal from fish or other marine animals could result in neurotoxic effects in this species.

Table 9.5 EU legislation on cadmium, lead, arsenic and mercury (total concentrations) in feed materials

	Products intended for animal feed	Maximum content (mg/kg)*
Cadmium	Feed materials of vegetable origin	1
	Feed materials of animal origin (except feedingstuffs for pets)	2
	Phosphates	10
	Complete feedingstuffs for cattle, sheep and goats (except young animals)	1
	Other complete feedingstuffs (except for pets)	0.5
	Mineral feedingstuffs	5
	Other complementary feedingstuffs for cattle, sheep and goats	0.5
Lead	Feed materials with the exception of:	10
	– green fodder	40
	– phosphates	30
	– yeasts	5
	Complete feedingstuffs	5
	Complementary feedingstuffs with the exception of:	10
	– mineral feedingstuffs	30
Arsenic	Feed materials with the exception of:	2
	– meal made from grass, from dried lucerne and from dried clover, and dried sugar beet pulp and dried molasses sugar beet pulp	4
	– phosphates and feedingstuffs obtained from the processing of fish or other marine animals	10
	Complete feedingstuffs with the exception of:	2
	– complete feedingstuffs for fish	4
	Complementary feedingstuffs with the exception of:	4
	– mineral feedingstuffs	12
Mercury	Feed materials with the exception of:	0.1
	– feedingstuffs produced by the processing of fish or other marine animals	0.5
	Complete feedingstuffs with the exception of:	0.1
	– complete feedingstuffs for dogs and cats	0.4
	Complementary feedingstuffs except:	0.2
	– complementary feedingstuffs for dogs and cats	

* Relative to a feedingstuff with a moisture content of 12%.
Source: EC Directive 2002/32/EC of 7 May 2002 on undesirable substances in animal feed.

For the other land animal species and poultry the mercury concentrations usually found in feedstuffs are well below the risk level for clinical toxicity. When it is possible to calculate it, the NOAEL in cattle (5 mg total mercury/kg feed), pigs (3.4 total mercury/kg feed) and poultry (2.2 total mercury/kg feed) are well below the maximum levels allowed in the EU (Table 9.4) and the normal concentrations found in feedstuffs.

9.4 Carry-over into food and human health risks

There is little information in the literature about experimental studies in livestock species given experimental doses of toxic metals at concentrations generally seen in animal diets to evaluate the transference to animal tissues.

In general, the carry-over of an orally administered compound to animal tissues and products (milk, eggs) is dependent on the absorption, distribution, metabolism and excretion/deposition of the compound and its eventual metabolites. These biological phenomena largely vary depending on the chemical form, are dose and/or time dependent (especially for some bioaccumulative elements like cadmium), and are influenced by other factors such as the interaction with other compounds (e.g. cadmium greatly interferes with essential elements such as copper and zinc).

9.4.1 Cadmium

Cadmium target tissues are liver and kidney, in these organs cadmium deposition being dose and time dependent (Linden, 2002), whereas in the muscle, cadmium deposition is very low and independent of the level of dietary cadmium exposure. Carry-over to milk and eggs is very low or absent (<0.05%; Blüthgen, 2000). Cadmium residues in the liver and kidneys of cattle (Sharma *et al*., 1979; Vreman *et al*., 1988; Smith *et al*., 1991; López-Alonso *et al*., 2000), sheep (Sharma *et al*., 1979; Hill *et al*.,1998; Lee *et al*., 1996), swine (Sharma *et al*., 1979; Hansen and Hinesly, 1979; Linden *et al*.,1999¸ López-Alonso *et al*., 2007) and poultry (Leach *et al*., 1979) fed standard diets (<0.5 mg/kg DM) were in most cases below the EU maximum admissible levels for animal products (i.e. 0.05, 0.5 and 1 mg/kg fresh weight for meat, liver and kidney respectively; EC, 2006). With increasing dietary cadmium exposure (1–5 mg/kg DM) cadmium residues in the liver and kidneys generally exceeded the cadmium permissible residues in all the farm animals, and with diets containing >5 mg/kg DM cadmium residues were one order of magnitude above these limits.

9.4.2 Lead

After a recent lead exposure, animal tissues with the highest residues are liver and kidney, whereas after chronic exposure lead accumulates in the

bone. Lead concentrations in milk are usually much lower than blood levels. Experimental studies in cattle (Vreman *et al*., 1988), sheep (Hill *et al*., 1998), and swine (Phillips *et al*., 2003) fed diets containing levels of lead varying between 15 and 25 mg/kg DM indicate that although residues in the liver, and especially in the kidney, were generally higher than in the control animals, they remained below the maximum permissible levels for animal products (0.1 and 0.5 mg/kg fresh weight for meat and offal, respectively; EC, 2006). In muscle lead residues were low and not significantly different from those in the control animals. At higher levels of lead dietary exposure (100 mg/kg DM) no significant changes in the tissue residues were found in the liver, kidney or muscle of sheep (Fick *et al*., 1976), although tissue levels significantly increased when dietary levels were 500 or 1000 mg/kg DM. Similar results were obtained for calves fed 100 mg/kg DM for 100 days (Dinius *et al*., 1973); liver and kidney contained 2.3 and 4.7 mg/kg fresh weight, but lead concentration in muscle tissue remained below the limit of detection. Blüthgen (2000) reported a carry-over percentage from feed to milk of 0.1–1%.

9.4.3 Arsenic

In mammalian species and poultry, inorganic arsenic is converted into methylated metabolites, which are rapidly excreted compared to other organic arsenic compounds. Hence the carry-over of arsenic compounds from feeds to edible tissues of mammalian species and poultry is very low. Thus although some caution is warranted, no major concerns on arsenic in animal feed from the human health point of view seem necessary (NRC, 2005).

Very limited information is available on experimental studies on arsenic carry-over on animal tissues. Arsenic residues in edible tissues (liver, kidney and muscle) of cattle (Vreman *et al*., 1986, 1988; Thatcher *et al*., 1985) sheep (Woolson, 1975; Veen and Vreman, 1985) and poultry (Proudfoot *et al*., 1991) fed standard or control diets (<2 mg/kg DM) are usually less than 0.01 mg/kg fresh weight. Similar arsenic residues are found in monitoring studies in animal products from various agricultural regions (Kramer *et al*., 1983; Salisbury *et al*., 1991; Kluge-Berge *et al*., 1992; Jorhem *et al*., 1991; Vos *et al*., 1986, 1987; López-Alonso *et al*., 2000, 2007). With increasing dietary arsenic exposure, arsenic residues significantly increase in all the analysed tissues compared with control animals. However, the absolute arsenic residue levels vary significantly depending on animal species, arsenic compounds and duration of exposure (Vreman *et al*., 1986, 1988; Eisler, 1994).

9.4.4 Mercury

The highest mercury levels are present in the skin, nails, hair and feathers; among the internal organs, kidneys contain the highest residues,

approximately 100-fold those in other tissues including liver or muscle (Clarkson, 1992).

Information related to carry-over of mercury into livestock animal tissues is very limited and no dose–response studies at the mercury concentrations usually found in feedstuffs are available concerning the transfer of inorganic or methylmercury into target species. Data from biomonitoring studies in livestock from relatively unpolluted areas (Korsrud *et al*., 1985; Vos *et al*., 1986; Jorhem *et al*., 1991; Niemi *et al*., 1991; Salisbury *et al*., 1991; Kluge-Berge *et al*., 1992; Falandysz, 1993a, b; Raszyk *et al*., 1996; Ulrich *et al*., 2001; López-Alonso *et al*., 2003a, 2007) indicate that total mercury concentrations in meat and meat products are generally below 10–20 μg/kg fresh weight, being below the quantification limit (generally 1–5 μg/kg fresh weight) in many liver and muscle samples. Based on these data, mercury residues in meat products do not appear to pose a relevant risk for human health, in fact the EU has not established a maximum mercury concentration for meat or meat products. However, the NRC (2005) points out that the levels of dietary and water mercury that are tolerated by livestock would result in tissue levels higher than 50 μg/kg, and consequently, standards for mercury levels in feed and water supplied to animals intended for human consumption in countries where maximum concentrations in animal feeds have not been established, should be based on tissue residue levels and not animal health concerns.

9.5 Brief review of detection methods

Traditionally the most common analytical methods for the determination of total toxic metal residues in feed, feedstuffs and animal tissues are based on atomic absorption spectrometry. Electrothermal atomic absorption spectrometry (ETAAS) has been the method of election for cadmium and lead, whereas cold vapour atomic absorption spectrometry (CV-AAS) is used for total mercury; either ETAAS or hydride generation atomic absorption spectrometry (HG-AAS) has been used for total arsenic determination. Standardized methods have been validated by official organizations such as the European Committee for Standardization (CEN) and the US Food and Drug Administration (FDA) in accordance with AOAC Guidelines. In addition, pressure (such as microwave) digestion procedures preceding AAS have been developed. In general, limits of detection and quantification of these methods are low enough to allow toxic metal concentrations/residues on most feed and feedstuffs and are well below the actual statutory limits.

In the last years the great advance of multielement analytical techniques such as inductively-coupled plasma atomic emission spectrometry (ICP-AES) and inductively-coupled plasma mass spectrometry (ICP-MS), which in addition provides lower detection limits than absorbance detection

methods, are displacing ASS, and official organizations are validating standard methods for multielement analysis.

For total mercury, a great enhancement of sensitivity (up to some orders of magnitude) and better selectivity has been obtained by cold vapour atomic fluorescence (CV-AFS) instead of atomic absorption.

Because of the great differences in toxicity of the more than 25 naturally occurring arsenic compounds (organoarsenicals) in the environment, analytical methods allowing a speciation of arsenic compounds have become available in food recently. HPLC-ICP-MS is the most commonly employed technique, although cation-exchange chromatography coupled with HG-AAS and atomic fluorescence spectrometry with hydride generation have also been used.

The great prevalence of methylmercury, the most toxic form of mercury, in marine feed has made it necessary to develop analytical methods for its quantification. Gas chromatography (GH) with both packed and capillary columns has been the most widely used technique for the separation of mercury compounds, while HPLC is increasingly being applied. CV-AAS, CV-AFS, ICP-MS and ICP-OES are appropriate for mercury detection.

9.6 Prevention of toxic metal contamination

Because of their great spread in the environment, the reduction of toxic metal contamination to undetectable levels in feed and in food is not possible; however, considerable efforts should be made to maintain toxic metal residues within acceptable levels.

Within animal feeds, monitoring programmes including for all categories of feedstuffs are especially relevant for cadmium and lead because of their prevalence in the terrestrial environment: toxic metal residues above the regulatory limits are frequently found in feedstuffs from polluted, especially industrialized and mining, areas, but also following the disposal of waste and sewage sludges. For arsenic and mercury, except in very localized areas, most residues come from feeds of fish origin, which makes it easier to control the entry of toxic residues in the food chain. Proper monitoring programmes, including sampling and analytical procedures, are encouraged in order to avoid contaminated feed commodities entering the food chain as well as to evaluate the temporal trend of toxic metal contamination in agriculture.

From the points of view of animal health and carry-over into human food, in addition to toxic metal concentrations in feed, interactions with other essential nutrients should be considered. As indicated, it is well documented that excessive copper and zinc supplementation (used as growth promoters) in livestock can lead to cadmium accumulations in the liver and kidney well above the statutory limits, so offal of livestock should be thoroughly monitored. On the other hand, excessive cadmium exposure has

been demonstrated to lead to secondary copper deficiency in ruminants growing up in cadmium-polluted areas, which could be minimized by supplementing livestock with the essential trace elements.

9.7 Regulatory controls

Currently the EU is the only government institution that has a comprehensive norm on maximum admissible concentrations or levels on feed and foods.

EU Directive 2002/32/EC of 7 May 2002 on undesirable substances in animal feed established maximum levels of arsenic, cadmium, lead and mercury in certain feeding stuffs covering feed materials of vegetable, animal and mineral origin, additives, pre-mixtures, complete and complementary feeding stuffs. Summarized information is presented in Table 9.5.

Criteria for sampling, sample treatment and methods of analysis for the official control of the maximum levels of these metals are laid down by Commission Regulation (EC) No. 333/2007 of 28 March 2007. Surveillance for residues of chemical elements in foods of animal origin is specified in Council Directive 96/23/EC.

Commission Regulation (EC) No. 1881/2006 of 19 December 2006 lays down maximum levels of cadmium and lead in certain foodstuffs and mercury in fish products (see also Chapter 10). These maximum values refer to the total amount of the respective elements. For arsenic no maximum level in food products is yet established.

9.8 Future trends

Proper monitoring programmes on a large scale, including sampling and analytical procedures, to evaluate toxic metal concentrations in feedstuffs and animal products for human consumption are encouraged. These data will provide comprehensive information on toxic metal residues in feedstuffs worldwide and will allow us to identify contaminated feed commodities so as to avoid them entering the food chain, to adopt measures to reduce toxic metal contamination in feedstuffs and to evaluate temporal trends of toxic metal contamination in agriculture. In relation to sampling, better characterization of feed categories or feed ingredients should be established by the corresponding food authorities to allow comparison. Sampling procedures, related to sample sizes, frequency and other parameters, should also be harmonized.

Although the maximum toxic metal concentrations in feedstuffs established by the EU seem to be low enough to guarantee animal health (the only exception appears to be organic mercury for cats and minks feed mostly fish-based diets) and to limit carry-over into food of animal origin

within the maximum admissible levels, there is a need for additional research on safe dietary levels of toxic metals for most livestock species to guarantee both animal and human health in other countries outside the EU.

In relation to analytical methods, although official methods are well established for total metal concentrations, the special toxicological relevance of some arsenic and mercury compounds makes it necessary to develop and validate official methods to specifically quantify them. For marine products the concentration of inorganic arsenic needs to be determined as a prerequisite for a comprehensive assessment of the potential animal health risks; more information on release of inorganic from organoarsenic compounds such as arsenosugars is also needed. Because methylmercury is the form of greatest toxicological concern, but also the predominant one in marine organisms, the analysis of methylmercury in feeds should be encouraged.

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10

Aquaculture feed contamination by persistent organic pollutants, heavy metals, additives and drug residues

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Abstract: In aquaculture compound feed is recognised as the major source of contaminants. When discussing contaminants in feed two classes of components need to be distinguished: components intentionally added to feeds, and undesirable substances that are present in feed or feed materials. A brief introduction to the characteristics of these different classes of contaminants and their sources and presence in aquafeed is provided. Moreover, the chapter discusses relevant EU legislation related to persistent organic pollutants, heavy metals, feed additives and drug residues. Special emphasis is given to the carry-over to edible tissue and measures to prevent contamination of feeds matrices.

Key words: persistent organic pollutants, heavy metals, feed additives, drug residues, MRL, fish feed.

10.1 Introduction

Fish and other seafood is a good source of nutrients such as proteins, marine *n*-3 fatty acids, selenium, iodine, and vitamin D. However, fish and seafood may also contain contaminants such as mercury or dioxins. In aquaculture compound feed is recognised as the major source of contaminants. In 2006 the world fisheries and aquaculture production was a total of 143.7 million tonnes, of which aquaculture accounted for 51.7 million tonnes (inland and marine production) (FAO, 2009). In intensive aquaculture, such as in the production of salmonids, cod, sea bass and sea bream, fish are fed compound feeds, while in extensive fish farming, such as farming of tilapia and carp, fish are mainly fed fodder. The estimated yearly production of compound feeds (2005) is approximately 23 million tonnes (Tacon and Hasan, 2007).

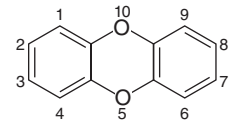
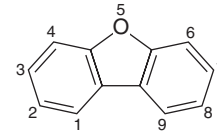
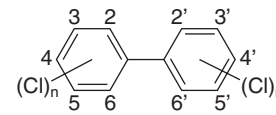
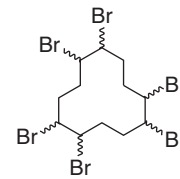
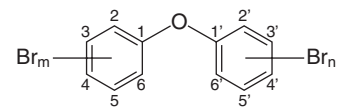
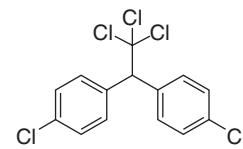
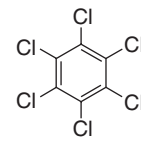
When discussing contaminated feed or contaminants in feed is it important to distinguish between two classes of components: components intentionally added to feeds, and compounds that are not intended to be found in feed. Chemical contaminants belong to the latter groups, and typically originate from the feed ingredients. Feed additives and veterinary drugs are added intentionally, and may be harmful if they are added in excessive concentrations or if residues of drugs are present above intentionally accepted concentrations in the fish. Compound feeds can get contaminated during processing and/or storage. This chapter will address contamination of compound feed (aquafeed) by persistent organic pollutants, heavy metals, additives and therapeutic drugs, and discuss their way into feed, carry-over to fish and ways of preventing contamination.

10.2 Persistent organic pollutants

Persistent organic pollutants (POPs) are non-polar, fat-soluble and persistent ubiquitous environmental pollutants. Most POPs are anthropogenic and are produced for industrial purposes (e.g. flame retardants used in textiles to prevent spread of fire, pesticides to protect crops against insect damage, polychlorinated biphenyls (PCBs) in plastics, etc.) or are by-products of industrial, and sometimes natural, processes (i.e. formation of dioxins by industrial combustion or forest fires and volcanic activity). POPs are often globally dispersed and readily bioaccumulate up the aquatic food chain; they can be found in elevated levels in wild fish. Oils obtained from pelagic fish species are the main source of POPs in aquafeeds and farmed oily fish such as Atlantic salmon (*Salmo salar*) (Berntssen and Lundebye, 2008).

Persistent organic pollutants that are of importance for aquafeeds and farmed fish include dioxins, dioxin-like polychlorinated biphenyls (PCBs), non dioxin-like PCBs, brominated flame retardants (BFRs), and organochlorine pesticides (OCPs) such as dichlorodiphenyltrichlorethane (DDT), hexacyclobenzene (HCB), toxaphene, aldrin, chlordane, endosulfan and hexacyclohexane (HCH). These are all halogenated (containing chlorine, bromine or fluorine ions) carbon structures, which can have many different chemical isoforms (congeners; for details see Chapter 8). Dioxin is a generic term given to two chlorinated ground structures, namely polychlorinated dibenzo-*para*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (commonly known as 'dioxins' or PCDD/Fs). Based on the number and position of the chlorine atom in the ground structure, a total of 210 congeners are possible (Table 10.1). Similarly, PCBs are organochlorine compounds consisting of two phenyl rings connected with a carbon bond, and a total of 209 different congeners theoretically exist (Table 10.1). A total of 12 PCBs have a 'dioxin-like' structure and biological effects (DL-PCBs); the other PCBs are considered non-dioxin-like PCBs of which six are used

Table 10.1 Overview of some of the most important persistent organic pollutants (POPs) present in aquafeeds and farmed fish

Contaminant group	Name	Structure
Dioxins	Polychlorinated dibenzo-*para* dioxins (PCDD)	 Chlorination at positions 1,2,3,4,6,7,8,9
Dioxins	Polychlorinated dibenzofurans (PCDF)	 Chlorination at positions 1,2,3,4,6,7,8,9
PBs	Polychlorinated biphenyls (PCB)	 Chlorination at positions 2,3,4,5,6
Brominated flame retardants	Hexabromocyclododecane (HBCD)	 Diastereoisometry of bromine atoms
Brominated flame retardants	Polybrominated diphenyl ethers (PBDEs)	 Bromination at positions 1,2,3,4,5,6
Organochlorine pesticides	Dichlorodiphenyltrichlorethane (DDT)	 *para*, *para*-chlorinated isoform of DDT
Organochlorine pesticides	Hexacyclobenzene (HCB)	 Only one isoform of HCB

as indicator PCBs. The BFRs represent a group of chemicals with a basic structure of aromatic rings that are brominated. The major BFRs in fish oils, aquafeeds and farmed fish are hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDEs). HBCD has three diastereomers (α, β, γ) of which α-HBCD is the dominant form in farmed fish (van Leeuwen *et al*., 2009), and PBDE has 209 theoretical congeners of which seven are used as indicators of overall PBDE contamination of feed ingredients, aquafeeds and farmed fish. The group of organochlorine pesticides is a diverse group of chemicals with many different chlorinated organic ground structures with different isoforms and/or breakdown products. The most dominant organochlorine pesticides present in Atlantic salmon aquafeeds are HCB, DDT, dieldrin, chlordane and toxaphene (Berntssen *et al*., 2010; Hites *et al*., 2004).

Besides their ability to accumulate in the marine food chain, POPs are also known to be potentially hazardous for humans. Consumption of oily fish, such as farmed salmon but also wild-caught fish such as mackerel, herring and eel, is an important source of human exposure (Bergkvist *et al*., 2008; De Mul *et al*., 2008; Fattore *et al*., 2008; Kvalem *et al*., 2009; van Leeuwen *et al*., 2009; Voorspoels *et al*., 2008). The overall exposure of dioxins and dioxin-like PCBs in adults with a normal seafood consumption has been shown to be under international recommended safe limits (e.g. Netherlands, De Mul *et al*., 2008; Norway, Kvalem *et al*., 2009; China, Zhang *et al*., 2008; Spain, Llobet *et al*., 2008). However, children in Sweden (Bergkvist *et al*., 2008) and Spain (Llobet *et al*., 2008) as well as adults with high seafood consumption in Sweden (Llobet *et al*., 2008) and Belgium (Sioen *et al*., 2008) have an estimated dietary dioxin and dioxin-like PCB intake that exceeds tolerable limits. The relatively high levels of POPs in oily fish have led to concern regarding POPs in farmed fish. Aquaculture feeds are the main source of POPs in farmed fish, and European maximum limits (MLs) for POPs in feed ingredients, aquafeeds and farmed fish products have been implemented to control the background levels of POPs in the food production chain and restrict human dietary exposure to these contaminants.

10.2.1 Sources of POPs in aquafeeds

The persistent and lipophilic character of most POPs causes them to bioaccumulate in oily fish, and fish oils obtained from pelagic fish species are the main source of POPs in aquaculture feeds (Jacobs *et al*., 2002). Aquafeeds for fatty fish such as Atlantic salmon have a high oil inclusion level (25–33%), and POP loads are higher compared to aquafeeds used for lean fish such as carp which have lower oil inclusion levels (10–15%). A large variation exists in the background levels of POPs found in fish oils. Some of the main factors determining background POP levels are (1) geographic origin of the fish species from which oils are obtained, (2) the season in which the pelagic fish are caught, and (3) the fish species (Berntssen and Lundebye,

2008). Fish oils obtained from pelagic fish from the South Pacific Ocean have lower levels of dioxins than fish oils produced from fish species from the North Atlantic Ocean, while fish oils produced from pelagic species in the Baltic Sea have high dioxin and dioxin-like PCB levels (Lundebye *et al*., 2004) (Table 10.2). Fish oil from pelagic fish species caught in the North Atlantic Ocean in winter have considerably lower levels of POPs (e.g. dioxins and PCBs) than fish oils obtained from fish caught in spring (Table 10.2). During early spring the lipid content decreases in the fish and consequently the concentration of dioxins and PCBs increases in the extracted oil (NORA, 2003). Fishmeal contains some oil (2–3%) that can be an additional source of POPs in aquafeeds. Other possible sources of dioxins in aquafeeds are contaminated feed additives such as anti-caking substances and/or mineral and vitamin mixtures (Eljarrat *et al*., 2002; Kim *et al*., 2007; Llerena *et al*., 2003). The latter are usually a minor contribution to the total load of dioxins in aquafeeds.

Farmed Atlantic salmon have traditionally been fed diets containing large amounts of fish oil and fishmeal. The steady increase in production volume in aquaculture of 8–10% a year (Tacon, 2004; Tacon *et al*., 2006), and demand for sustainable fish farming that relies less on marine fish ingredients such as fish oil and meal, have resulted in increasing use of alternative proteins and oils in aquafeeds. In this respect, vegetable oils and plant protein have been proposed as sustainable alternatives to fish oil and fishmeal (e.g. Torstensen *et al*., 2008). Most plant ingredients have lower levels of POPs than marine ingredients, and use of plant ingredients lowers the load of POPs in aquafeeds (e.g. Bell *et al*., 2005; Berntssen *et al*., 2005, 2010; see Section 10.2.3). Incorporation of novel protein and oil sources as feed ingredients potentially exposes farmed fish to contaminants that may otherwise be of limited significance. Pesticides are of concern with regards to the use of vegetable oils as alternative fish feed ingredients, and these ingredients may also contain elevated levels of polyaromatic hydrocarbons (PAHs) (Berntssen *et al*., 2010). Vegetable oils can contain higher levels of

Table 10.2 Levels of dioxins and dioxin-like PCBs (WHO-TEQs ng/kg wet weight) in different fish oils used in aquafeeds with variation in season and region of origin

Season and geographic region	Dioxins	Dioxin-like PCBs
Berntssen *et al*., 2005, North Atlantic		
Capelin (*Mallotus villosus*), winter	6.1	6.8
Capelin (*Mallotus villosus*), spring	7.5	8.7
Lundebye *et al*., 2004		
Species and season unknown, South Pacific	1.1	5.9
Species and season unknown, Baltic	14	16

Source: after Pickova *et al*. (2010).

PAHs than retail fish oils (Dennis *et al*., 1991), and technological processes such as direct fire drying of grain, oilseeds or oil are potential contamination sources of PAHs in vegetable oils (EFSA, 2007; Speer *et al*., 1990).

10.2.2 Legislation

The European Union (EU) has implemented maximum limits (MLs) for potentially hazardous substances, including environmental contaminants such as POPs, throughout the entire food production chain. Legislation exists on MLs in feed ingredients, feeds and food including edible parts of farmed fish. The MLs aim to protect animal welfare, the environment and consumer safety.

Legislation on dioxins and dioxin-like PCBs is based on the sum of 29 different PCDD, PCDF and PCB congeners. These congeners have a structure and potentially toxic action that resembles the PCDD with four chlorine atoms in the outermost positions (2,3,7,8-TCDD). This dioxin congener is considered to be the most toxic, and the other congeners have been assigned toxic equivalency factors (TEF) that express their toxicity relative to 2,3,7,8-TCDD. A total of 17 PCDD/F congeners (seven PCDD and 10 PCDF), and 12 PCBs that have dioxin-like structure and toxic properties, have been allocated a TEF value. The concentration of dioxins and dioxin-like PCBs is expressed in toxic equivalents (TEQ), where a toxic equivalency factor (TEF) is applied to the concentration of the individual congeners and summed to generate the total TEQ in a mixture of the 29 congeners. The TEFs established in 1997 by the World Health Organization (WHO) have been widely used for human risk assessment (Van den Berg *et al*., 1998) by government bodies, and these TEFs were re-evaluated in 2005 (Van den Berg *et al*., 2006) and are used in legislation from 2012. The European Commission's former Scientific Committee on Food established a tolerable weekly intake for dioxins (including dioxin-like PCBs) of 14 pg WHO-TEQ/kg body weight for adults; JECFA (the Joint FAO/WHO Expert Committee on Food Additives) has set a provisional monthly intake of dioxins and dioxin-like PCBs of 35 pg WHO-TEQ; whereas the WHO has set a tolerable daily intake of 1–4 pg WHO-TEQ/kg body weight. The EU has established maximum limits for dioxins (total TEQ for the 17 congeners with TEFs) and for the sum of dioxins and dioxin-like PCBs (total TEQ for the 29 congeners with TEFs) in fish feed, fish oil; fish protein hydrolysates containing more than 20% fat, and fish, other aquatic animals, their products and by-products (with the exception of fish oil), and fish oil; these limits are given in Table 10.3 taken from Commission Directive 2006/13/EC. These limits are based on the WHO-TEFs from 1997. The maximum permitted level (in WHO-TEQ) for dioxins alone, and for the sum of dioxins and dioxin-like PCBs, in muscle meat from fish for human consumption are 3.5 and 6.5 pg WHO-TEQ/g fresh weight, respectively (Commission Regulation (EC) No. 1259/2011), with the exception of muscle meat from eel, for which the maximum levels are 3.5 and 10 pg

Table 10.3 Current EU maximum limits of POPs in feed ingredients (here only fish oil and fishmeal are given), feed, and farmed fish

	Fish oil	Fishmeal	Fish feed	Farmed fish
Dioxins (pg WHO1998-TEQ/g)				
PCDD/F	6	1.25	2.25	4
PCDD/F + DL-PCBs	24	4.5	7	8
Organochlorines (ng/g)				
Aldrin/dieldrin	200	10	10	
Endrin	50	10	10	
α-HCH	200	20	20	
γ-HCH (lindane)	2000	200	200	
HCB	200	10	10	
Sum chlordane (*cis–trans* chlordane, oxychlordane, *cis–trans* nonachlor)	50	20	20	
Heptachlor (heptachlor and heptachlor A)	200	10	10	
Sum toxaphene (26 + 50 + 62) (camphechlor)	200	50	50	
Sum endosulfan (α + β + sulfate)	100	100	100	
sum DDT (*op*- and *pp*-DDT, DDD and DDE)	500	50	50	

WHO-TEQ/g fresh weight for dioxin alone and for summed dioxin and dioxin-like PCBs, respectively. Commission Regulation EC No. 199/2006 was replaced by 1259/2011, which is based on WHO-TEFs from 2005 as apposed to WHO-TEFs from 1997.

Non-dioxin-like PCBs include a group of 197 different congeners, of which six have been allocated as indicator PCBs for the non-dioxin-like PCB group. The ML of indicator PCBs (sum of PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180) in muscle tissue of most fish species is 75 ng/g, and in eel is 300 n/g (Commission Regulation EC No. 1259/2011). Similarly for PBDE, seven indicator PBDEs have been indentified to assess PBDE contamination of feed ingredients, feeds and farmed fish. No official EU legislation currently exists for PBDEs in food and feed products. For organochlorine pesticides maximum limits are established for feed ingredients and aquafeeds, while no specific EU limits exist for organochlorine pesticides in seafood. The EU maximum limits for aldrin, dieldrin, toxaphene, chlordane, DDT, endosulfan, endrin, heptachlor, hexachlorobenzene and hexachlorocyclo-hexane in feedingstuffs are given in Commission Directive 2002/32/EC.

10.2.3 Carry-over of POPs to edible tissue and prevention of contamination

Most POPs present in aquafeeds readily accumulate in the fat deposits of farmed fish. Maximum steady-state levels of most POPs are not expected

to be reached within a normal production cycle (Berntssen *et al*., 2005). The duration of the feeding period, in addition to feed concentrations, is important in determining the final POP levels in farmed Atlantic salmon. Other factors that determine POP contamination levels in farmed fish are concentration in feed, growth rate and feed utilisation (Berntssen *et al*., 2007), with a lower fillet contamination in fish with higher growth rates and better feed utilisation. Approximately 29% of the consumed PCDD/Fs and 52% of the DL-PCBs are retained in the edible part of market-size cultured rainbow trout (*Oncorhynchus mykiss*) (Isosaari *et al*., 2002; Karl *et al*., 2003). The retention of DL-PCBs is higher than that of PCDD/Fs, which causes relatively higher levels of DL-PCBs compared to dioxins in muscle than in feed (e.g. Berntssen *et al*., 2007). For PBDEs the skinned fillet accumulates 42–59% of the sum of PBDE consumed (Isosaari *et al*., 2005). Biotransformation of higher-brominated PBDEs (e.g. penta-BDE-99 and hepta-BDE-183) into lower-brominated congeners (e.g. tetra-BDE-47 and hexa-BDE-154) is an important factor in the carry-over of dietary PBDE congeners (Stapleton *et al*., 2004; Tomy *et al*., 2004). Similarly, the α-diastereoisomer which is recalcitrant to bioisomerisation and the dominant diastereoisomer in fish, is biotransformed from both the β- and the γ-diastereoisomers in juvenile rainbow trout (Law *et al*., 2006). In farmed rainbow trout, the dietary retention of sum chlordane (*cis-trans* and oxychlordane) and sum toxaphene in the edible part of market-size fish was approximately 33 and 27%, respectively (Karl *et al*., 2002). In commercially reared sea bream (*Dicentrarchus labrax*) biomagnification of DDTs and HCB from feed to muscle has been shown to occur (Serrano *et al*., 2003). In cultured Asian seabass (*Lates calcarifer*), 15% of DDT and its metabolites are partitioned in muscle (Bayen *et al*., 2005). The dietary retention of a DDT metabolite (DDE) and dieldrin in whole large mouth bass (*Micropterus salmonids floridanus*) after prolonged exposure was approximately 20–25% and 32–35%, respectively, with lower levels in muscle compared to gonads and liver (Muller *et al*., 2004, 2005). Contaminants that originate from vegetable oils such as PAH and the pesticide endosulfan are less persistent than the contaminants mentioned above, and biological breakdown plays an important role in the low carry-over from feed to fillet of these contaminants (e.g. Berntssen *et al*., 2008, 2010a).

There are three main strategies that singly or in combination prevent or reduce POP contamination of aquafeeds. These include (1) sourcing of marine ingredients with low background levels of POPs, (2) replacing marine ingredients with ingredients that have very low levels of POPs, such as plant ingredients, and (3) the decontamination of marine feed ingredients.

Selective use of marine fish oils with naturally low levels of dioxins and dioxin-like PCBs, such as oil obtained from fish in the Pacific Ocean, has been reported to reduce the levels of dioxins, and to a lesser degree dioxin-like PCBs in farmed Atlantic salmon (Isosaari *et al*., 2004; Lundebye *et al*.,

Table 10.4 Level of sum dioxins and dioxin-like PCBs (sum PCDD/F + DL-PCB ng WHO-TEQ/g ww) in Atlantic salmon and rainbow trout

Reference	Species	Strategy	Sum PCDD/F + DL-PCB	
			Diets	Fillet
Hites *et al.*, 2004	Norwegian Atlantic salmon	Surveillance data 2003–2004		2.4
Måge *et al.*, 2008	Norwegian Atlantic salmon	Surveillance data 2008		1.2
Friesen *et al.*, 2008	Canadian Atlantic salmon	Surveillance 2003		1.4
Bell *et al.*, 2005	Atlantic salmon	Fish oil replacement during a full life cycle	0.78	0.68
Berntssen *et al.*, 2005	Atlantic salmon	Fish oil replacement during a full life cycle	0.33	0.3
Friesen *et al.*, 2008	Atlantic salmon	Fish oil replacement, 6 months		0.25
Berntssen *et al.*, 2010	Atlantic salmon	Combined fish oil and fishmeal replacement, 12 months	0.94	0.74
Sprague *et al.*, 2010	Atlantic salmon	Decontaminated fish oil 3 months	0.45	0.34
Berntssen *et al.*, 2010b	Atlantic salmon	Decontaminated fish oil during full seawater cycle	0.44	0.21
Oo *et al.*, 2007	Rainbow trout	Combined fish and fishmeal replacement, 3 months	0.1	0.2
Drew *et al.*, 2007	Rainbow trout	Combined fish and fishmeal replacement, 5 months	0.1	0.1

2004). Market-size Atlantic salmon fed on diets based on fish oils of Pacific origin for a period of 30 weeks had a mean sum of PCDD/F and DL-PCB levels of 2.9 ng WHO-TEQ/kg ww, which was not lower than the typical level found in Norwegian farmed Atlantic salmon fillets on the market in 2003–2004 (approximately 2.5 ng WHO-TEQ/kg ww: Hites *et al*., 2004).

The increased use of terrestrial oils (both vegetable and animal oils) has been shown to reduce levels of PCBs and dioxins in Atlantic salmon and rainbow trout (Bell *et al*., 2005; Berntssen *et al*., 2005, 2010a; Drew *et al*., 2007; Friesen *et al*., 2008; Oo *et al*., 2007) (Table 10.4). The full substitution of fish oils with terrestrial oils results in dioxin and dioxin-like PCB levels in farmed Atlantic salmon around 0.3 ng WHO-TEQ/kg ww (Berntssen *et al*., 2010b). In standard aquaculture practice, both fishmeal and oil are replaced to a certain extent to produce sustainable aquafeeds; however, a certain level of fish oil is included to guarantee optimal production (Torstensen *et al*., 2008). Use of combined substitution of fish oil and fishmeal by vegetable oil and plant protein in feed for Atlantic salmon gave a mean sum PCDD/F and DL-PCB of 0.74 in salmon fillets versus 2.4 ng WHO-TEQ/kg ww for Atlantic salmon reared on fish oil and meal-based feeds (Berntssen *et al*., 2010). Combined replacement of fish oil and meal in rainbow trout aquafeeds led to fillet dioxin and dioxin-like PCB levels of ~0.2 ng WHO-TEQ/kg ww versus ~1.2 ng WHO-TEQ/kg ww for rainbow trout fed fish oil and meal-based feeds (Drew *et al*., 2007; Oo *et al*., 2007).

The use of decontamination of fish oil and fishmeal techniques is another way to reduce POP loads in aquafeeds while maintaining high levels of marine nutrients such as very long-chain omega-3 fatty acids. Various techniques have been developed and are currently available to remove POPs from fish oils. The use of active carbon efficiently removes PCDD/F (99%) whereas this method is less efficient for decontaminating oils with regards to DL-PCB (73%), and PBDEs are not removed at all (Maes *et al*., 2005; Oterhals *et al*., 2007; Usydus *et al*., 2009). The formation of PAHs in vegetable oils can be prevented by selecting processing techniques (EFSA, 2007) or direct decontamination with the use of active carbon (Larsson *et al*., 1987). Other techniques such as counter-current supercritical CO_2 extraction will remove both PCCD/F and DL-PCB with a high (~95%) efficiency (Kawashima *et al*., 2009), and short-path distillation will reduce PCDD/Fs, DL-PCBs, PBDEs and OCPs (Berntssen *et al*., 2006).

10.3 Heavy metals

Metals in the aquatic environment originate from natural or anthropogenic sources; metals are released by volcanic activity and from weathering of bedrocks, or by human activities such as mining activities, incineration of waste or agricultural use. The heavy metals cadmium, mercury and lead are released to the environment by burning of fossil fuels and by smelting

of ore and other metal extraction processes. Mercury and cadmium are used in parts of the world as pesticides and fertilisers, and thereby introduced to the environment. The metalloid arsenic is also spread to the environment through its use in agriculture in some countries as a constituent of pesticides, growth promoters and wood-preserving agents. The heavy metals and arsenic are non-essential elements and have no biological function in fish.

Metals and metalloids occur in inorganic or organic forms, as free ions, as small organometallic compounds or as part of larger biomolecules. The chemical form of a metal/metalloid has great significance for its toxicity, bioavailability and carry-over from feed to animal. In the marine environment mercury mainly occurs as methylmercury; inorganic mercury is methylated to methylmercury by microorganisms in sediments and the water column. Methylmercury is readily bioavailable and bioaccumulates up the marine food chain. Methylmercury is the major form of mercury in fish and fillet, constituting more than 70% of the total mercury present (Bloom, 1992; Storelli *et al*., 2002, 2005). Mercury is toxic to most species depending on dose and chemical form, as methylated forms are more toxic than inorganic mercury (Clarkson, 1997).

The opposite is the case for arsenic where inorganic arsenic is much more toxic than methylated forms of arsenic (Mandal and Suzuki, 2002). More than 30 arsenicals have been identified in the marine environment to date (Francesconi and Kuehnelt, 2002). The major form of arsenic in marine animals, including fish, is arsenobetaine (Cullen and Reimer, 1989; Edmonds and Francesconi, 1987; Francesconi and Edmonds, 1994; Francesconi and Kuehnelt, 2002), which often comprises more than 95% of total arsenic levels (Francesconi and Edmonds, 1997). Arsenobetaine is considered innocuous to fish and humans (Kaise and Fukui, 1992; Kaise *et al*., 1985; Sakurai *et al*., 2004).

10.3.1 Sources of heavy metals in aquafeeds

Metals have a high affinity for e.g. sulphur and tend to bind to carbonate, phosphate or sulphur groups in proteins and are hence often associated with the protein fraction. The marine protein fraction of fish, i.e. fishmeal, is the major source of metals in fish feed; fishmeal contains much higher levels of cadmium, lead and mercury than fish oil where levels are low, often below the limit of quantification (Table 10.5, Måge *et al*., 2008). Both fishmeal and fish oil contain relatively high background levels of arsenic, and both ingredients contribute to the high level of arsenic seen in fish feed (Table 10.5, Måge *et al*., 2008). Other feed ingredients may also contribute to the level of metals found in fish feed. Plant oil, e.g. rapeseed oil, contains arsenic and lead where levels up to 0.5 and 1.1 mg/kg, respectively, have been found (Table 10.5, Måge *et al*., 2008). Other alternative marine feed ingredients such as krill are a potential source of elements such as copper,

Table 10.5 Concentrations (mg/kg, mean and range) of arsenic, cadmium, lead and mercury in fish feed and feed ingredients. Data are from the Norwegian surveillance programme on feed for fish and other aquatic organisms

	n	Arsenic	Cadmium	Lead	Mercury
Fish feed	22	4.0 (2.3–8.7)	0.28 (0.10–0.61)	0.09 (<0.04–0.48)	0.042 (<0.029–0.12)
Fishmeal	20	6.5 (1.9–13.0)	0.50 (0.14–1.70)	0.08 (<0.04–0.30)	0.13 (0.05–0.24)
Fish oil	10	8.3 (4.6–11)	<0.03	<0.04	<0.03
Plant oil (rapeseed oil)	10	0.13 (<0.03–0.5)	<0.03	0.15 (<0.04–1.1)	<0.03

Source: Måge *et al*. (2008).

fluorine and cadmium in fish feed (Moren *et al*., 2006, 2007). Another route into aquafeeds is through feed ingredients, e.g. mineral mixtures of essential elements, which are often supplemented to animal feed and can be contaminated with heavy metals.

10.3.2 Legislation

In 2000 the European Commission declared in a White Paper that 'The safety of food from animal origin begins with safe animal feed' (EU, 2000), and since then European feed and food legislation has been revised several times. European feed legislation aims to protect the animals, the consumer, the user and the environment, and sets maximum permitted levels for a range of undesirables substances in feed ingredients and complete feedingstuffs (Commission Directive 2002/32/EC and amendments). The current maximum content for arsenic in fish feed is 10 mg/kg and 25 mg/kg (12% moisture content) for feed ingredients of marine origin, with the exception of seaweed and products thereof where the level is 40 mg/kg (Commission Directive 2009/141/EC). The Directive recognises that inorganic arsenic is the most toxic arsenical and that marine feed ingredients mainly contain organic arsenic compounds. The maximum levels are based on the level of total arsenic, since standardised routine analytical methods for the detection of inorganic arsenic are currently lacking. Authorities can request documentation showing that the level of inorganic arsenic in marine feed ingredients is below 2 mg/kg (Commission Directive 2009/141/EC). For cadmium and lead the current maximum permitted content in fish feed is 1 and 5 mg/kg, respectively, while for feed ingredients it is 2 and 10 mg/kg, respectively (Commission Directive 2005/87/EC). The current maximum content of mercury in fish feed and marine feed ingredients is 0.2 and 0.5 mg/kg, respectively (Commission Directive 2010/6/EC).

10.3.3 Carry-over of heavy metals to edible tissue and prevention of contamination

Carry-over describes the transfer of a given chemical compound from feed to animal tissues. The carry-over of a contaminant from aquafeeds to fish is of importance with regards to fish welfare, and the carry-over to edible parts of fish is of relevance for seafood safety.

The carry-over of arsenic and mercury largely depends on their chemical form, as both arsenobetaine and methylmercury are readily accumulated in fish, while the retention of inorganic arsenic and mercury is low. The retention of arsenobetaine is high in muscle following oral exposure; approximately 40% and 50% retention has been reported for yellow-eyed mullet (*Aldrichetta forsteri*; Francesconi *et al*., 1989) and Atlantic salmon (Amlund and Berntssen, 2004), respectively. Although the retention in muscle is high, the carry-over from feed to fillet seems to be low, being approximately 8% and 15% for Atlantic salmon and Atlantic cod (*Gadus morhua*), respectively, after three months of exposure (Amlund *et al*., 2006). Methylmercury is mainly accumulated in muscle following oral exposure. Berntssen *et al*. (2004) found that > 83% of body burden in Atlantic salmon was found in the fillet following exposure to graded levels of methylmercury. The carry-over of methylmercury from feed to fillet is high; for Atlantic cod the estimated carry-over was approximately 40% (Amlund *et al*., 2007).

The retention of dietary cadmium and lead is generally low (less than 10%) in whole fish, and the metals are mainly accumulated in the intestine, liver and kidney, while little is accumulated in muscle tissue. The accumulation seems to depend on the concentration in the feed and the duration of exposure. Research has shown that transfer of cadmium from salmon feed to salmon fillet is very low (2–6%). Rainbow trout fed 150 mg cadmium/kg for one month showed elevated levels in kidney and liver but not in muscle tissue (Handy, 1992). The carry-over of lead from feed to fillet is low, and in rainbow trout lead is mainly retained in the intestine, bone, kidney and liver (Alves and Wood, 2006; Alves *et al*., 2006).

Inclusion of alternative feed ingredients in traditional fish oil and fishmeal such as vegetable oil and plant protein can alter the levels of arsenic and heavy metals in aquafeeds. Vegetable oil contains considerably lower levels of arsenic than fish oil (Table 10.5) and the inclusion of plant oil will likely reduce the final level of arsenic in the feed produced.

10.4 Feed additives

In contrast to POPs and heavy metals, feed additives are deliberately added to fish feed, but can be considered contaminants if present at concentrations higher than authorised. They are typically authorised for specific species (sometimes for all species, such as minerals and vitamins) and

they have specified purity and criteria for conditions of use. Feed additives are products used in animal nutrition for improving the quality of feed and consequently the quality of food of animal origin, or to improve the animals' performance and health, for example to enhance the digestibility of feed materials. There are different categories of additives as described in Table 10.6, depending on their function. Technological additives do not influence technological aspects of the feed directly by influencing the nutritional value of the feed, but they may do so indirectly by improving handling or hygiene characteristics. Functional groups of technological additives include preservatives, antioxidants, emulsifiers, stabilisers, thickeners, gelling agents, binders, substances for control of radionuclides, anticaking agents, acidity regulators, silage additives and denaturants (e.g. mycotoxins; for details see Chapter 13).

10.4.1 Legislation

Council Regulation (EC) No. 1831/2003 established new rules for the authorisation, supervision and labelling of feed additives, replacing Council Directive 70/524/EEC. All feed additives placed on the market in the European Union must be authorised under Regulation 1831/2003/EC. The corresponding details for implementation in terms of the assessment and authorisation of feed additives are given in Commission Regulation (EC) No. 429/2008. This regulation includes an application form (Annex I), a description of the studies requested to prepare a dossier (Annex II) and a specific part (Annex III). This specific part is dedicated to different additive categories and functional groups, feed additives already used in food, additives used only for 'minor species', additives only used in pet food, modifications and re-evaluation of additives authorised under Council Directive 70/524/EEC. The EFSA conducts risk assessment regarding food and feed safety for the European Union; in particular EFSA's FEEDAP Panel

Table 10.6 Categories and functions of feed additives

Category	Function
Technological additives	Influence the technological aspects of the feed
Sensory additives	Improve the palatability of a feed by stimulating appetite
Nutritional additives	Supply a specific nutrient required by the animal for optimal growth
Zootechnical additives	Improve the nutrient status of the animal by enabling more efficient use of the nutrients present in the feed

evaluates safety to the target animal, the consumer, the user (farmer) and the environment in addition to the efficacy in the target species. EFSA has published several guidance documents (available at EFSA's website) to clarify its approach to the scientific risk assessment of feed additives. If an additive is authorised by the European Commission, the conditions of its use are included in the feed additives register which is a public document. See Section 10.7 for further sources of information regarding legislation related to feed safety.

10.4.2 Carry-over of feed additives to edible tissue

Details regarding the assessment of safety of the use of additives for the consumer are given in a technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (EFSA, 2008).

The assessment of consumer safety of feed additives is based on experimental studies that investigate the metabolic fate and residues of the additives in the target species and laboratory animals as well as the potential toxicity of the additive in *in vitro* biological systems and laboratory animals. These data are used to establish an acceptable daily intake and to propose maximum residue limits (MRLs) and withdrawal periods as management tools, taking into account food consumption (see EFSA's Concise Food Consumption Database) which also allows the identification of high consumers and of consumers with special dietary preferences.

Many of the feed additives originally authorised under Council Directive 70/524/EEC do not have MRLs as yet, for example synthetic antioxidants that are authorised for use as feed additives in the European Union, including ethoxyquin (EQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), octylgallate and propylgallate. EQ and BHT are generally added to fishmeal and fish oil, respectively, to limit lipid oxidation. Fishmeal needs to be stabilised to prevent spontaneous combustion during overseas transport and storage. Addition of either BHT or EQ at concentrations of at least 100 mg kg^{-1} at the time of shipment is used to prevent such combustion (IMO, 2003). In contrast to BHT and BHA, EQ is only authorised as a feed additive, but not as a food additive in the EU. However, EQ has been shown to be carried over from feed to farmed fish (Bohne *et al*., 2008; Lundebye *et al*., 2010), and also to poultry tissues and eggs (Hobsen-Frohock, 1982). Hence it is expected that MRLs will be established for synthetic antioxidants and other feed additives in the reauthorisation process.

10.4.3 Prevention of contamination

An entirely new class of compounds has recently been added to the list of feed additives, the so-called mycotoxin binding and ameliorating compounds or denaturants, commonly known as mycotoxin binders. The potential benefits and the use of these compounds are discussed in Chapter 13.

10.5 Drug residues

Fish in aquaculture may be subject to a wide range of diseases, some of which are of an infectious nature mediated by viruses, fungi and bacteria, or by internal and external parasites. For infectious diseases where treatment is available, veterinary drugs may be administered by baths or by inclusion in the feed. Currently there are no effective veterinarian drugs for the treatment of viral infections in aquaculture, and fungi are not considered a problem in the seawater phase thus antifungal agents are not administered via the feed. Therefore, only drugs against infection by bacteria and external or internal parasites may be added to the feed. A more thorough discussion on drug consumption in aquaculture and the subsequent residue control may be found in Lunestad and Samuelsen (2008).

Information on types and amounts of therapeutic agents used in aquaculture throughout the world is not easily obtainable, since only a few countries provide reliable and accessible statistics on consumption of these drugs. As an example, Norway has provided statistics from aquaculture since the late 1980s (Bangen *et al*., 1994). In 2010 the following amounts of antibacterial agents were added to fish feed in Norway: oxolinic acid 308 kg, florfenicol 275 kg, oxytetracycline 10 kg and lincomycine/spectinomycine 57 kg. In 2010 these sea-lice agents were added to medicated fish feed: 22 kg emamectine benzoate, 1839 kg diflubenzuron and 1080 kg teflubenzuron. In addition 11 kg of the anti-cestode agent praziquantel were applied in feed this year. All figures are given as quantities of active component and updated statistics for Norway are available at http://www.fhi.no/.

The use of quinolones and other antibiotics included in the feed remains unrestricted in aquaculture in some countries with growing aquaculture industries, such as China and Chile. For example, in Chile, statistics indicate that 10 to 12 metric tons of quinolones are used annually in human medicine, and approximately 100 to 110 metric tons of these antibiotics are used in veterinary medicine annually, most of them in aquaculture (Cabello, 2006). In Chile the use of flumequine, a fluoroquinolone used exclusively in aquaculture, has increased from approximately 30 metric tons in 1998 to close to 100 metric tons in 2002 (Bravo *et al*., 2005).

10.5.1 Sources of drug residues in aquafeeds

Veterinary drugs are intentionally included in the feed for treating infectious diseases. Common concentrations of antibiotics in fish feed may be in the range of 1 to 5 g/kg feed and for sea-lice agents from 0.5 to 2 g/kg feed. The global application of veterinary drugs for prophylactic purposes is not known. However, one could argue that common treatment in aquaculture during an outbreak is predominantly prophylactic, since the non-diseased fish will eat more medicated feed compared to diseased fish.

In some cases carry-over of veterinary dugs seems to be unavoidable. Thus, Commission Directive 2009/8/EC gives maximum levels of unavoidable carry-over to non-target feed of coccidiostats and other agents added to poultry feed to cope with the problems of single-cell parasites. These levels are set following the ALARA (As Low As Reasonably Achievable) principle. Commission Directive 2009/8/EC permits a carry-over rate to non-target feed of 1–3%. This principle does not apply to aquaculture feed, and residues of therapeutic agents are not to be found in non-target feed.

10.5.2 Carry-over of drug residues to edible tissue

In contrast to other contaminants, veterinary drugs are given to fish or other organisms in aquaculture, to provide the intended dose and thereby the desired therapeutic effect. Since low concentrations of drug residues may be found in treated animals for extended periods post-therapy, is it necessary to establish acceptable drug residue concentrations in fish for human consumption. These acceptable concentrations are expressed as MRLs, and are based on a toxicological evaluation of the substance, combined with information on food consumption patterns. Regulation (EC) No. 470/2009 described the current EU Community procedure for the establishment of MRLs for veterinary medicinal products in food of animal origin. The established MRLs are listed in Commission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacological active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Consumption of food with drug residues below the MRL should, by sufficient safety margins, not pose a health risk to the consumer. The MRLs are given for each substance either for all food-producing species or for groups of related species such as salmonid fish. The establishment of MRLs is aided by the European Medicines Agency (EMA, previously abbreviated EMEA) (http://www.emea.eu.int/). The EMA coordinates the evaluation and supervision of medicinal products throughout the European Union, and performs the scientific evaluation of drugs to be used in aquaculture. Information on specific drugs may be found on the web page of EMA (http://www.ema.europa.eu/ema/index.jsp). Appropriate withdrawal periods are set in order to reach the MRLs. These periods are defined as the minimum time to elapse between terminated medical treatment and harvest of the organism in question, and are specific for each drug, species and sometimes water temperature. For each substance the period to reach an acceptable residue level should be prolonged with an extra safety margin in case of individual fish-to-fish variations in drug elimination.

Drug residues in fish are also covered by Council Directive 96/23/EC. According to this directive each member country should conduct annual surveillance for a number of undesirable substances in all food-producing species. At least one sample for each 100 tons produced should be

examined, and samples should be collected according to specified plans, taking into account the production patterns of each country. The components specified in Council Directive 96/23/EC include legally applied drugs, drugs that are not permitted as hormones and other growth promoters, as well as organic and inorganic pollutants, mycotoxins and certain dyes. Each member country has to submit reports on their activity on an annual basis. Council Directive 96/23/EC represents an important and standardised means of drug residue control among the members of the European Community.

10.5.3 Prevention of cross-contamination

To avoid unintended contamination of aquafeeds and thereby aquaculture products by veterinarian drugs, several measures need to be combined. The internal control of the producers of the medicated feed, the fish farming enterprise, the slaughter house, fish processors and retailers have to maintain a system for documentation of the quality and safety of their products. One important control point in this system is the absence of drugs in non-target feed and fish.

The key aspect in avoiding cross-contamination of non-target fish feed by therapeutics is the introduction of reliable traceability systems, including clear labelling and the control of all ingredients used in the production line.

10.6 Future trends

The supply of traditional marine feed ingredients is stable while the demand for fish feed continues to increase, hence there is a need for alternative feed ingredients, such as plant protein or vegetable oil. As discussed above, the substitution of traditional feed ingredients with new ingredients can reduce the level of several contaminants in aquafeeds. However, the use of alternative feed ingredients can also introduce new contaminants, e.g. pesticides. New environmental pollutants continue to be found in the marine environment, and these pollutants can be characterised as emerging contaminants in marine feed ingredients. Over the last five years, there have been several cases of contamination of feed through the use of contaminated feed ingredients.

10.7 Sources of further information and advice

More information regarding contaminants in aquaculture can be found in:

Berntssen M H G and Lundebye A-K (2008), 'Environmental contaminants in farmed fish and potential consequences for seafood safety', in Lie Ø, *Improving Farmed Fish Quality and Safety*, Cambridge, Woodhead, 39–70.

The following websites provide information regarding feed and food safety:

- The Joint FAO/WHO Expert Committee on Food Additives (JECFA): http://www.who.int/ipcs/food/jecfa/en/index.html (accessed 13 February 2010)
  http://www.fao.org/ag/agn/agns/jecfa_index_en.asp (accessed 13 February 2010)
- The Joint FAO/WHO Meeting on Pesticide Residues (JMPR): http://www.who.int/ipcs/food/jmpr/en/ (accessed 11 April 2011)
- The European Food Safety Authority (EFSA): http://www.efsa.europa.eu/ (accessed 13 February 2010)
- The European Commission, Directorate General for Health and Consumers:
  http://ec.europa.eu/dgs/health_consumer/index_en.htm (accessed 13 February 2010).

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COMMISSION DIRECTIVE 2006/13/EC of 3 February 2006 amending Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed as regards dioxins and dioxin-like PCBs.

COMMISSION DIRECTIVE 2009/8/EC of 10 February 2009 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels of unavoidable carry-over of coccidiostats or histomonostats in nontarget feed.

COMMISSION DIRECTIVE 2009/141/EC of 23 November 2009 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards

maximum levels for arsenic, theobromine, *Datura* sp., *Ricinus communis* L., *Croton tiglium* L. and *Abrus precatorius* L.

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COMMISSION REGULATION (EU) No. 37/2010 of 22 December 2009 on pharmacological active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

COMMISSION REGULATION (EC) No. 1259/2011 of 2 December 2011 amending Regulation No. 199/2006 setting maximum levels for certain contaminants in foodstuffs as regards dioxin and dioxin-like PCBs.

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11

Mycotoxin contamination of animal feed

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Abstract: This chapter first describes mycotoxin contamination of raw feed materials, mixed feeds and concentrates. Thereafter potential animal health risks are discussed addressing all major farm animal species, fish and pet animals. Finally, the carry-over of mycotoxins into animal-derived food products, such as milk, eggs and meat, by mycotoxins is presented and the risk for human consumers that is associated with such residues is explained. The last part of this chapter is devoted to the current legal provisions and the methods to control the level of feed contamination by mycotoxins.

Key words: mycotoxin, feed, animal toxicity, residues in edible tissues, milk and eggs.

Note: This chapter contains updated material from Chapter 12, 'Controlling mycotoxins in animal feed,' by H. Pettersson, published in *Mycotoxins in Food: Detection and Control*, eds N. Magan and M. Olsen, Woodhead Publishing Limited, 2004, ISBN: 978-1-85573-733-4.

11.1 Introduction

Almost all agricultural crops become infested by moulds (fungi imperfecti) either during the growing season, or during preservation, transport and storage. Agricultural practices aim to control or reduce fungal growth, but these measures cannot eliminate fungal growth entirely. Fungal invasion can reduce crop yield as well as altering the nutritional value of the crop; however, it is the formation of mycotoxins which remains the major hazard for human and animal health. Cereals and grains of poor quality, and with visible mould damage, are disregarded for human consumption and graded as animal feed. The risk of mycotoxin contamination of crops and raw feed materials differs, depending on the type of crop, the geographic area and climate, the use of fungicidal agents, harvest conditions, preservation and storage conditions. The type of feed material is the most important determinant of the mycotoxin profile and the quantities of mycotoxins that can be expected in the diet of the animals. The risk of adverse effects by

mycotoxins on animals depends on the quantity of contaminated feed material actually consumed and the mycotoxin concentration at the time of feeding. Animal species differ in sensitivity towards mycotoxins and may respond to exposure with different toxic effects, such as reduced performance or immunosuppression and impaired fertility, the latter being observed often at a later stage.

11.2 Routes of mycotoxin contamination: raw feed materials

Animals are mainly exposed to mycotoxins through the consumption of contaminated feed. The occurrence of the most common mycotoxins in different feed materials, concentrates and mixed feeds, varies significantly. Another way in which animals can be exposed to mycotoxins is through the inhalation of fungal spores and debris of fungal mycelia. This material is known to induce irritation and inflammation of the upper airways. In addition, it is well known that particularly fungal spores (conidia) can contain high amounts of mycotoxins, contributing to overall exposure. Some fungal species, such as *Aspergillus fumigatus*, can also invade animal tissues and produce mycotoxins at the site of infection.

Raw materials for feed are commonly divided into cereals and cereal by-products, oilseeds and their by-products, leguminous seeds, roots and tubers, and animal by-products, all of which are used for the production of compound feeds. In addition, animals may consume pasture grass and green crops, silage and haylage, hay and straw in varying quantities, in particular ruminants and horses. Mycotoxins are commonly associated with various different feedstuffs, through their production during the growing season or storage, as summarized in Table 11.1.

11.2.1 Cereals and cereal by-products

Cereals and cereal by-products are among the main feed resources used in developed countries; they are used mainly for pigs, poultry and cattle. Cereals are normally grown, harvested, preserved and stored at the farm where they will be used as feed or are obtained from local feed mills. In principle, culturing and harvesting of cereals for feed is the same, or similar, to the methods used for cereals intended for human consumption. Barley, oats and triticale are more commonly used as feed cereals, but wheat and maize are also important crops used in animal diets.

Cereals (monocotyledons) are infected in particular by the fungal species of the genus *Fusarium* during the flowering period. These fungi produce, as major toxins, deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), fumonisins (FB), HT-2 and T-2 toxins. DON may be found at very high levels in wheat and barley, especially in years when *Fusarium*

Table 11.1 Feedstuffs and their associated mycotoxins at different stages of production. Toxins printed in bold type are relatively more common in the feedstuff

Feed raw material	Mycotoxins produced	
	In field during culturing	Preservation and storage
Cereals and by-products		
Barley	**DON**, NIV, ZEA, HT-2, T-2	**OTA, Afla, Cit**
Maize	DON, Fum, ZEA	**ZEA, Afla**
Maize gluten	**DON, Fum, ZEA**	**ZEA, Afla**
Millet		
Oats	**DON, NIV, HT-2, T-2**	OTA, Cit
Rice		**Afla**, Sterig, OTA
Rye	**Ergot**	**OTA**
Sorghum		Afla
Wheat	**DON, NIV**, ZEA, Ergot	**OTA**, Afla, Cit
Wheat bran	**DON, NIV**	**OTA**
Oilseed by-products		
Coconut meal		**Afla**
Cottonseed meal	**Afla, Tenua**	**Afla**
Groundnut meal	**Afla**, ZEA	**Afla**, OTA, Cit
Linseed meal		
Palm kernel meal		**Afla**
Rapeseed meal		
Soya bean meal		Afla
Sunflower meal		
Leguminous seeds		
Beans		OTA
Peas		OTA
Roots and tubers		
Cassava	ZEA	**Afla**
Fodder beet		
Sugar beet pulp		
Silages		
Grass		**Roq, Pat, PR, ZEA**
Leguminous		**Roq, Pat, PR, ZEA**
Maize	**DON**, ZEA, Afla	**Roq, Pat, PR, ZEA**
Hay		
Straw	DON	ZEA, Sat
Pasture		
Grass	ZEA, DON, Ergot	
Rye grass	Spor, Lol, Pasp	
Tall fescue	Ergot	
Red clover	Slaf	
Lupine	Phom	

Abbreviations: Afla = aflatoxins; Cit = citrinin; DON = deoxynivalenol; Ergot = ergotalkaloides; Fum = Fumonisin; HT-2 = HT-2 toxin; Lol = lolitrems; NIV = nivalenol; OTA = ochratoxin A; Pasp = paspalitrem; Pat = patulin; Phom = phomopsin; PR = PR-toxin; Roq = Roquefortin C; Sat = satratoxin; Slaf = slaframine; Spor = sporidesmin; Sterig = sterigmatocystin; T-2 = T-2 toxin; Tenua = tenuazonic acid; ZEA = zearalenone.

is at epidemic levels in North America and Europe. Triticale can also be highly contaminated with DON. HT-2 and T-2 toxins have been found in remarkably high concentrations in oats grown in north-west Europe during most years since the beginning of the twenty-first century (Edwards *et al.*, 2009). Oats have also been identified as being highly contaminated with DON and NIV in recent years. Maize (and the products thereof) grown in temperate climate zones contains predominantly DON and ZEN, whereas maize from subtropical and tropical areas is more often contaminated with fumonisins and aflatoxins, especially after drought stress or insect damage.

In the northern hemisphere, small grains are normally harvested with a moisture content above the level that is generally considered as safe (i.e. 13 to 15% moisture). Preservation (post-harvest drying) is needed in these cases to be able to store the grains safely. Drying with heated air, as applied to food-grade cereals, can be used for the purposes of preservation, but this method is often too expensive when applied to high-moisture grains. In addition, many farmers prefer to dry the grains only to a moisture content of approximately 15%, due to the better milling conditions and the consumption by animals at this moisture level. Slow drying with ambient air for longer periods is still in use; however, it has been shown that this can increase the risk of contamination by storage moulds, for example by the formation of ochratoxin A (OTA) by *Penicillium verrucosum* (Jonsson and Pettersson, 1999; Holmberg *et al.*, 1990).

Acid preservation and airtight storage are alternative methods used to stabilize feed cereals with higher moisture content. Among the acids that can be used to prevent mould growth during storage, propionic acid is most widely used to suppress mould growth and mycotoxin formation. In contrast, formic acid has been shown to promote, for example, aflatoxin formation (Holmberg *et al.*, 1989). With airtight storage there is the remaining risk that, under practical conditions, airleakage can occur. Some fungal species, such as *Penicillium roqueforti*, are able to grow under micro-aerobic conditions, hence this is one of the most common mould species in airtight stored cereals.

Maize gluten feed

Maize gluten feed consists of gluten, bran and germ from the wet milling of maize. Studies on the fate of mycotoxins during the wet milling of maize have shown that the mycotoxins are concentrated in the bran and germ fractions. Analysis of maize gluten feed has also revealed a high occurrence of mycotoxins such as aflatoxin, ZEA and DON (Scudamore *et al.*, 1997, 1998; Veldman *et al.*, 1992; Olsen *et al.*, 1986). Maize gluten feed is used mostly in concentrates for dairy cattle and in mixed feeds for poultry.

Wheat bran and feed meal
Wheat bran and feed meal are produced in the mills as by-products during the milling of wheat for baking flour. They are popular feedstuffs mainly used in mixed feeds for pigs and poultry. Studies on the fate of mycotoxins during the milling process have shown that they are concentrated in these milling fractions (Lee *et al*., 1987; Seitz *et al*., 1985, 1986; Chelkowski *et al*., 1982). Hence, feedstuffs produced from wheat milling by-products are considered as products at risk.

Oat feed
Oat feed or its by-products are derived from oat processing and consist of oat hulls, small kernels and debris. Dehusking and milling studies have shown that nearly all major *Fusarium* toxins (DON, T-2 and HT-2 toxins) are found in the hull fraction (Scudamore *et al*., 2007). The trichothecene contamination of oat-based feeds can therefore be very high.

Cereal grain screenings
Grain screenings are the residues from the cleaning of cereals and comprise broken pieces of grain, small grains and dust from the outer layers of the grain. The mycotoxin concentrations in small kernels have been found to be much higher than in the cleaned grains (Trenholm *et al*., 1991; Lee *et al*., 1992; Chelkowski *et al*., 1982). Cereal grain screenings have also been found to contain high mycotoxin levels (Ross *et al*., 1991; Murphy *et al*., 1993).

Draff
Draff consists of the insoluble grain material from the brewer's or distiller's malt after processing. Mycotoxins may be found in the grains used for malting and can also be produced during the malting process. They may therefore end up in both draff and beer, and have been found to be relatively common in both products (Baxter, 1996). The leftover material from the production of ethanol fuel also belongs to this category, and is known as distiller's dried grains with solubles (DDGS), although it is often fed to animals as a slurry (see also Chapter 21). Maize and wheat are mainly used for the production of ethanol fuel. Draff and DDGS are used for cattle and pig feed.

11.2.2 Oilseed by-products

Oilseed cakes and meals are the residues remaining after the removal of the greater parts of the oil from oilseeds. The residues are rich in protein and most are valuable feedstuffs for all farm animals. Cakes and meals produced from soya bean, groundnut, cottonseed, rapeseed, sunflower, coconut, palm kernel, linseed and sesame seed are used. The oilseeds

produced in tropical and subtropical areas are often infected by *Aspergillus flavus* or *A. parasiticus* during both plant growth and storage, and are consequently contaminated with aflatoxins. The high water activity in oilseeds in relation to moisture content can also promote growth of fungi and the production of aflatoxins. Oilseed cakes and meals are often transported by ships from the place of production to the country where the goods will be consumed. During the transport they can easily become moist with continued fungal growth and toxin production. Cakes and meals of groundnut, cottonseed, copra and palm kernel are often reported as being contaminated with aflatoxin (Pittet, 1998; Scudamore *et al*., 1997). Soy meal from some subtropical countries has occasionally also been contaminated with aflatoxins. These oilseed cakes and meals are often used in concentrates for dairy cattle; this use has been found to be responsible for the occurrence of aflatoxin M1 in milk.

11.2.3 Leguminous seeds

There are only a few reports of mycotoxin occurrence in leguminous seeds. However, OTA has often been found in beans (*Phaseolus*) and peas (*Pisum*) (Domijan *et al*., 2005; Åkerstrand and Josefsson, 1979). Beans and peas from tropical and subtropical areas have also occasionally been found to be contaminated with aflatoxins. Moulds with the potential to produce mycotoxins have, on the other hand, been found frequently on both beans and peas. Lupine seeds can be contaminated with phomopsin, but this toxin is more often associated with forage based on lupine (Lacey, 1991).

11.2.4 Roots and tubers

Cassava is frequently found to be contaminated with aflatoxins. They are mainly produced during drying and storage in countries with a humid climate (Essono *et al*., 2009; Thieu *et al*., 2008). ZEA has also been found frequently in cassava, though this may have been produced during the growing season, rather than during storage.

Mycotoxin contamination of potatoes, sugar and fodder beets have rarely been reported, although the *Fusarium* species, with the potential to produce mycotoxins, can frequently be found in the isolated dry roots of beets and potatoes (Burlakoti *et al*., 2008; Bosch and Mirocha, 1992).

11.2.5 Silages

The green crops from pasture grass or grass/clover mixtures are preserved through ensiling. The crop is pre-dried and compressed, sometimes with the addition of formic acid, molasses or bacterial cultures, in order to promote ensiling. It is then packed and stored under anaerobic conditions in silos,

clamps or big plastic-coated bales. The anaerobic condition is vital for the ensiling process. If the crop is not tightly packed, air will be trapped in between the crop material. This is common in the middle of big bales produced by certain big bale presses (Skaar, 1996). *Penicillium roqueforti*, which is quite acid-tolerant and micro-aerophilic, will invade the middle of the big bale or the poorly compressed areas. These fungi can produce several toxins, but roquefortin C and mycophenolic acid have mostly been found in silage (Auerbach *et al*., 1998; Schneweis *et al*., 2005). If air leaks into the silo when opened or through the plastic of the big bale during storage (for example, due to damage of the plastic by birds), the silage will become mouldy and deteriorated. *Fusarium* moulds will often start to grow, and produce toxins, in wet crop material where the surface is exposed to air leakage. Other toxigenic moulds found in deteriorated silage are *Aspergillus fumigatus*, *Byssochlamys nivea* and *Paecilliomyces variotti* (Skaar, 1996) followed by the production of gliotoxin and patulin, respectively.

Maize silage

It is now common practice for the whole maize crop to be chopped, packed and stored anaerobically in silos. Fungi and mycotoxins from the pre-harvest stage will enter the silage. *Fusarium* and *Fusarium* toxins are commonly found in maize silage produced in northern regions (Driehuis *et al*., 2008b), whilst *Aspergillus flavus/parasiticus* and aflatoxins can be found in silage produced in subtropical and tropical areas.

Fungi may continue to grow and produce toxins as long as there is oxygen available. As with grass silage, maize silage is sensitive to secondary invasion by storage moulds, such as *Penicillium roqueforti* and others. Silages are mainly used for cattle, but are also given to sheep and horses.

11.2.6 Pasture

Pasture is an important feed source used by grazing animals. The pasture could be cultivated to a certain extent, or it could remain natural. Cultivated pastures often consist of different grasses and legume species, grown together. They can be infected by fungi already from seed, as well as during the growing period, especially in the overripe stages. Several different mycotoxins can be formed in plants and have been shown to cause mycotoxicoses in the grazing animals. Grasses and grass seeds from certain regions and of certain varieties that are found in pastures, such as tall fescue or rye grass, may contain endophytic fungi (*Neotyphodium* spp., previously denoted *Acremonium*), which produce typical mycotoxins (such as ergovaline and lolitrems). Legumes, including red clover, can in certain regions be infected by fungi producing slaframine (the causative agent of slobber's syndrome).

More information on the occurrence of mycotoxins in forage (pasture, silage and hay) is given in the reviews by Lacey (1991) and Fink-Gremmels (2005).

11.2.7 Hay and straw

Pasture (grasses) can also be dried, pressed and made into hay bales. Nowadays, the grass is mainly dried in strings at the lay before pressing. It is turned over during the drying process and can easily be contaminated with soil containing fungal spores, consequently rainfall during the drying process can trigger mould growth. Pressing of bales from still moist hay has the highest risk for mould growth. *Aspergillus fumigatus* is very common in mould-deteriorated hay (for further information on this see Fink-Gremmels, 2005).

Straw

Straws from cereals are collected and pressed into bales after harvest. Straw is used as bedding for pigs, horses and cattle. Pigs and horses can consume a large quantity of their bedding straws. Straw from scab-affected cereals should not be used for animals, since levels of ZEA and DON can be relatively high in this material. The practice of straw being left in the field and unprotected against rain involves a high risk of infection with *Stachyobotrys atra*, which can produce satratoxin. Stachyobotrytoxicoses have mainly been reported as present in grasses in Eastern Europe and France (Le Bars and Le Bars, 1996; Hintikka, 1977; Rodricks and Eppley, 1974), but can also occur in other countries.

11.3 Routes of mycotoxin contamination: mixed feeds and concentrates

Mixed feed and concentrates are mostly produced at feed mills, but some big farms also have the knowledge and equipment to produce them. The concentrates are often mixed with cereals (milled or crushed) and fed to pigs and poultry, or to ruminants together with forage.

The concentrates are normally produced by mixing feedstuffs with high protein and fat content, to get a mixture with energy and digestible protein suitable for use together with cereals and forage for specified animals. Oilseed meals, mainly soya and rapeseed but also cottonseed, groundnut, palm kernel and copra, are often used. Leguminous seeds (beans, peas and lupin seeds), animal protein (fish meal, blood meal, meat by-product, milk protein) and fat of vegetable and animal origin, can also be included. Maize and maize gluten meal may also be included, but this is more common in mixed feed for poultry and pigs. Cereals and cereal by-products are otherwise included to make up the final mixed feed. Amino acids, phosphates, minerals, trace elements and vitamins are also included to secure the nutritional value in the mixed feed. The composition of the concentrates and mixed feeds is dependent on the nutritional demand of the animal and the price and availability of the individual single feedstuff. Computer programs are used by the feed manufacturers to optimize the price and composition.

Low cost, and sometimes the associated low quality, feedstuffs will be promoted by this computerized optimization used in the feed mills.

Commercial mixed feeds and concentrates are often extruded and pelleted. Propionic or formic acid can also be added to reduce the risk of mould and salmonella growth in the feed. Such processing of the feeds will kill or reduce fungi and bacteria. Microbiological examination, using conventional methods, of such processed feed can no longer reveal low hygiene quality of the feed and its ingredients before processing. The mycotoxins are, however, stable and can be analysed. Many of the individual feedstuffs used in concentrate and mixed feed production are products at risk from mycotoxin contamination.

11.3.1 Aqua feed

Feeds for the aquaculturing of fish or crustaceans are industrially made by mixing different raw and plain feed ingredients; this mixture is then processed into small pellets. Plant-based ingredients from agricultural production have increased during recent years as a replacement for fish meal. They pose a higher risk of introducing mycotoxins into the diets than animal-based ingredients (Spring and Fegan, 2005). Maize gluten, wheat, soybean meal, lupine meal and draff are common new ingredients in fish feed. Maize gluten, wheat and draff, in particular, may contribute high levels of DON, ZEA and aflatoxins.

11.3.2 Pet food

The commercial production of extruded mixed feed for dogs and cats is of high importance. Wheat and maize meals, as well as soy meal, are important constituents in modern animal food production; therefore the products may be contaminated with mycotoxins. High levels of DON, OTA and aflatoxins have been found in dry food for dogs, which in some cases have caused intoxications. Conserved (canned) meat meals for cats and dogs have been found to be contaminated with mainly low levels of OTA and aflatoxins. The worldwide contamination of pet food with mycotoxins has been reviewed by Leung *et al*. (2006).

11.3.3 Food leavings

Production leftovers and food products which have passed their expiry date are often used as feed for pigs, and in private households for dogs. These food leavings are easily infested with *Penicillium* moulds. Many different penicillium toxins have been identified in such material, among them the tremorgenic mycotoxins roquefortin C and penitrem A (Rundberget *et al*., 2004). OTA, aflatoxins and sterigmatocystin have also been encountered in spoiled food materials.

11.4 Animal health risks

Exposure of animals to mycotoxins through the feed will affect both their performance and their health. High to medium concentrations of the mycotoxins in the feed will often produce acute and specific toxic effects and symptoms in the animals. However, low levels of mycotoxins, which are more common in feed and are often fed to animals for longer periods, will mainly result in economic losses due to lower performance, chronic toxic effects (reproductive toxicity) or reduced resistance to bacterial infections as many mycotoxins act as immunosuppressants.

11.4.1 Pigs

Pigs are considered to be the farm animal which is most affected by mycotoxins. This is probably due to their high consumption of cereals, which are often contaminated by mycotoxins, and their single stomach (monogastrics). The toxic effects in pigs fed with the most common mycotoxins are described below, and the lowest adverse effect levels in feeding studies are summarized in Table 11.2.

Deoxynivalenol and other trichothecenes

Barley, wheat and maize are common cereals in pig rations and they are often infested in the field by *Fusarium* species, resulting in contamination with high levels of deoxynivalenol (DON, also known as vomitoxin) in particular.

DON at high levels (12–20 mg/kg) in the feed for pigs will cause complete feed refusal and vomiting (Young *et al*., 1983; Forsyth *et al*., 1977). Feeding trials with low DON levels (0.6–2 mg/kg) in the diet have shown decreased feed consumption and weight gain in the pigs (Overnes *et al*., 1997; Bergsjö *et al*., 1992; Young *et al*., 1983). With naturally infected feed at slightly higher levels (3–6 mg DON/kg) epithelial lesions in the stomach have been observed. Reduced feed intake is generally considered as the most sensitive toxic effect; a temporary reduction in feed intake has even been observed in pigs given such a low level as 0.35 mg DON/kg from naturally contaminated grains (Friend *et al*., 1982).

No signs of altered feed intake or body weight, and no vomiting, were observed in pigs given 2.5 or 5 mg nivalenol (NIV) per kg feed for three weeks (Hedman *et al*., 1997). Altered kidney appearance and macroscopic changes with hemorrhagic damage and thickening of the gastrointestinal tract were seen in both groups, with higher frequency in the group fed 5 mg NIV/kg. A reduction in feed intake was found in another pig fed with 5.8 mg NIV per kg feed (Williams *et al*., 1994; Williams and Blaney, 1994).

Mainly high doses of T-2 toxin have been used in feeding studies with pigs. Reduced feed intake and weight gain have been found in pigs given 5–10 mg T-2 toxin/kg feed in these studies (Harvey *et al*., 1990, 1994; Rafai

Table 11.2 Lowest toxic effect levels in growing pigs fed different mycotoxins in feeding studies

Toxin	Lowest effect level (ppm)	Toxic effect	Reference
Aflatoxin B1	0.2–0.4	Microscopic lesions in liver	Edds 1979, Miller *et al.* 1981
	0.3–1.0	Reduced weight gain, enlarged thymus and spleen	Panangala *et al.* 1986, Lindemann *et al.* 1993
Ochratoxin A	0.2	Enzyme inhibition in kidney	Krogh *et al.* 1988
	1	Kidney damage, degenerated tubuli	Krogh *et al.* 1988
	0.2–2	Reduced growth	Madsen *et al.* 1982a, b, Tapia and Seawright 1985, Harvey *et al.* 1989
	2–2.5	Reduced immune response	Harvey *et al.* 1992, Verma *et al.* 2004
Deoxynivalenol	0.35–4	Reduced feed consumption and growth	Friend *et al.* 1982, Overnes *et al.* 1997, Bergsjö *et al.* 1992, Young *et al.* 1983, Trenholm *et al.* 1991
	>15	Vomiting	Young *et al.* 1983, Forsyth *et al.* 1977
Nivalenol	2.5	Mucosa and kidney changes	Hedman *et al.* 1997
	5	Reduced feed consumption, growth and leucocyte number	Hedman *et al.* 1997, Williams *et al.* 1994, Williams and Blaney 1994
T-2 toxin	0.5	Reduced immune defence	Rafai *et al.* 1995b
	1–2	Reduced growth and feed intake	Rafai *et al.* 1995a, Friend *et al.* 1992
Zearalenone	0.20–0.47	Enlarged vulva and uterus	Friend *et al.* 1990, Bauer *et al.* 1987, Döll *et al.* 2003, Gutzwiller *et al.* 2009
Fumonisin	1	Proliferation connective tissue in lungs	Zomborszky-Kovács *et al.* 2002a, b
	5	Increased serum Sa:So ratio	Riley *et al.* 1993, Zomborszky-Kovács *et al.* 2002a, b
	>15	Changed blood enzyme levels, reduced weight gain	Motelin *et al.* 1994

et al., 1989; Rafai and Tuboly, 1982). Rafai *et al*. (1995b) observed a reduction in feed intake in piglets fed 0.5 mg or more purified T-2 toxin/kg feed for three weeks. Decreased antibody response and plasma leukocyte count were also found in those pigs.

A more detailed description of the toxic effects from trichothecenes fed to pigs can be found in the reviews by Eriksen and Pettersson (2004) and Rotter *et al*. (1996).

Zearalenone – maize

Zearalenone (ZEA) causes estrogenic effects in pigs, growing female pigs being particularly sensitive. Intoxication of pigs is commonly observed in pigs fed high amounts of contaminated maize. Adverse effects which have been reported in sexually mature animals are ovarian atrophy, prolonged oestrus, persistent corpora lutea, pseudopregnancy, decreased fertility, stillbirth, implantation failure, and delivery of weak piglets (Kuiper-Goodman *et al*., 1987).

Early studies suggested that concentrations above 1 mg/kg feed would produce clear estrogenic effects (Mirocha and Christensen, 1974). In more recent studies, even concentrations between 0.05 and 0.4 mg ZEA/kg feed have been seen to produce estrogenic effects in young female pigs (Bauer *et al*., 1987; Lusky *et al*., 1997).

Diekman and Green (1992) reviewed ZEA effects on pig reproduction. They observed that only concentrations above 2 mg ZEA per kg in feed may affect the fertility of gilts, which also was confirmed in the study of Gutzwiller *et al*. (2009). The effects of ZEA in pigs and other animals were reviewed in detail by Diekman and Green (1992) and Fink-Gremmels and Malekinejad (2007).

Fumonisins – maize

Fumonisin exposure of pigs is mainly through contaminated maize, such as corncob mix or maize screenings.

Fumonisin B1 (FB1) intoxication of pigs is characterized by pulmonary, cardiovascular and hepatic changes. Lethal pulmonary oedema and hydrothorax have been seen in pigs given feed containing more than 12 mg FB1/kg (Haschek *et al*., 2001). Levels as low as 1 mg FB1/kg in feed in one study produced proliferation of the connective tissue, primarily around the lymphatic vessels, as well as in the subpleural and interlobular connective tissue, but the alterations were not accompanied by clinical signs (Zomborszky-Kovács *et al*., 2002a, b). Fumonisins affect the Sa : So (sphinganine : sphingosine) ratio. Both Riley *et al*. (1993) and Zomborszky-Kovács *et al*. (2002a) found an increased Sa : So ratio in serum from pigs fed FB1 concentrations ≥5 mg/kg feed. The literature data on the effect of FB1 on growth performance are inconsistent, but it was estimated that 21 mg/kg would cause a 5% reduction in weight gain (Dersjant-Li *et al*., 2003).

Ochratoxin A – cereals
Barley and wheat intended for pig feeding are often insufficiently dried in northern Europe, especially when ambient air is used after wet harvesting conditions. Growth of *Penicillium verrucosum*, even at relatively low temperatures, can produce high amounts of ochratoxin A (OTA) during storage.

When OTA is present in the pig ratio for a long periods, this will cause kidney damage. Pale and enlarged kidneys are frequently observed at slaughter; they are rejected and sometimes so is the whole carcass. Chronic nephropathy has been observed from dietary levels of 1 mg/kg diet for two years (Krogh and Elling, 1977; Elling, 1979, 1983; Meisner and Krogh, 1986; Krogh *et al*., 1988). Dietary levels as low as 0.2 mg OTA/kg diet for 90 days have, in a study, caused a reduction in renal activity of cytosolic phosphoenolpyruvate carboxykinase and γ-glutamyl transpeptidase accompanied by decreased kidney function.

Aflatoxins – cassava + rice + maize
From early feeding studies and field cases, the effect of aflatoxin in pigs has been summarized as induced liver damage and icterus, gross hemorrhages in many parts of the body, ataxi, stunting, and immunosuppression. In graded feeding studies with young growing pigs, clear toxic effects have first been seen when the toxin level in the feed is more than 300 μg AFB1/kg (Southern and Clawson, 1979; Edds, 1979; Sisk *et al*., 1968). Reduced weight gains, depression, anorexia and icterus have been observed at those low levels, but microscopic hepatic lesions are the main effect at low levels (Miller *et al*., 1981). Dersjant-Li *et al*. (2003) have reviewed seven feeding studies with low AFB1 concentrations in pig feed, and have calculated the average effect of AFB1 on pig performance. It was found that 300 μg AFB1/kg feed caused on average a 5% reduction in the body weight gain compared to control.

11.4.2 Poultry
The susceptibility of poultry to the different mycotoxins, which they are exposed to mainly through cereals and oilseed by-products, varies a lot. Broilers are often less sensitive compared to turkeys and ducks. The toxic effects of the most common mycotoxins are described below and the lowest adverse effect levels in feeding studies are summarized in Table 11.3.

Aflatoxin – maize
Broiler chickens are susceptible to the toxic effects of aflatoxin B1 (AFB1) in their feed. Several cases of aflatoxicosis in broiler chickens have been reported. Decreased growth rate, feed efficiency, enlarged liver, spleen and pancreas, and a regression of the Bursa of Fabricius have been seen in chickens fed ≥1 ppm AFB1 (Richardson *et al*., 1987; Smith and Hamilton, 1970). Fatty liver damage and hypocarotenoidemia are observed at low

Table 11.3 Lowest toxic effect levels in broiler chickens fed different mycotoxins in feeding studies

Toxin	Lowest effect level (ppm)	Toxic effect	Reference
Aflatoxin B1	0.2–0.6	Liver damage, decreased T-lymphocytes and cell-mediated immunity, reduced immune defence	Ghosh *et al.* 1990, Thaxton *et al.* 1974
	0.5–1	Reduced weight gain, feed efficiency, liver damage, enlarged spleen, pancreas and hypocarotenoidemia	Verma *et al.* 2004, Richardson *et al.* 1987, Smith and Hamilton 1970, Doerr *et al.* 1983, Edds 1973
Ochratoxin A	0.2–0.3	Histological changes in kidney	Krogh *et al.* 1974, 1976
	0.5	Reduced T-lymphocytes and cell-mediated immunity	Chang *et al.* 1979, Singh *et al.* 1990
	0.5–1	Reduced growth	Huff *et al.* 1975, Krogh *et al.* 1976, Golinski *et al.* 1983, Ayed *et al.* 1991
	1	Macroscopic changes of kidneys	Krogh *et al.* 1974, 1976
Deoxynivalenol	9	Reduced liver and gizzard weight	Kubena *et al.* 1985
	16	Reduced feed consumption and growth	Kubena *et al.* 1987, 1988, 1989, Kubena and Harvey 1988, Harvey *et al.* 1991
	50	Mucosa erosions	
Nivalenol	3	Reduced feed consumption and growth, gizzard erosions	Hedman *et al.* 1995
T-2 toxin	0.4–1	Mucosa erosion	Wyatt *et al.* 1972, 1973
	2	Reduced feed consumption and growth	Wyatt *et al.* 1972, 1973
Zearalenone	300	Increased growth rate, comb, ovary and bursa	Speers *et al.* 1971
Fumonisins	20–75	Changed Sa:So ratio, decreased cholesterol	Weibking *et al.* 1993, Henry *et al.* 2000

AFB1 concentrations in maize-fed broilers (Doerr *et al*., 1983; Edds, 1973; Smith and Hamilton, 1970). The relationship between dietary AFB1 and decreased weight gain in broilers has been compared using 10 feeding studies; it was calculated that a 5% decrease in weight gain can be expected at 1 mg AFB1/kg feed (Dersjant-Li *et al*., 2003).

Turkeys are more sensitive to AFB1 than broiler chickens, and they were involved in the first described aflatoxicosis cases in England in 1960. The growth rate of turkeys fed 0.5 mg AFB1/kg feed was suppressed in two different studies by 19% and 18%, respectively (Kubena *et al*., 1991; Hamilton *et al*., 1972). The mortality rate was low at 0.5 mg/kg but increased to 88% at 1 mg/kg. The relative weights of the kidney, pancreas and gizzard were increased, but the weights of the liver and the Bursa of Fabricius were decreased.

Ochratoxin A – cereals, beans

Chickens and hens are, like other poultry, exposed to ochratoxin A (OTA) mainly through cereals and cereal products mixed into their feed. Some may also come into contact with peas and beans, which are also often contaminated by OTA.

Feeding trials with graded concentrations (0.3–18 ppm) of purified OTA in feed for broiler chickens have been performed by several researchers (e.g. Huff *et al*., 1975; Krogh *et al*., 1976; Golinski *et al*., 1983). Growth was retarded by 0.5–1.0 mg/kg feed in most of the studies. OTA is thus one of the most potent mycotoxins in this aspect for chickens, though it is mostly known for causing kidney damage. This effect can be seen as kidneys are enlarged and pale when chickens are given 1.0 ppm OTA and higher concentrations in their feed. The related higher water consumption was also found in some studies.

Other reported toxic effects when chickens are fed more than 2 ppm are decreased bone strength, skeletal changes, lower absorption of carotenoids, anemia, glycogen accumulation in breast muscle, and an increase in intestinal ruptures during processing.

OTA also impairs the blood coagulation in chickens fed 2 ppm and more, by elevating the prothrombin times. An increase in the susceptibility for bruising to appear on the thigh and leg during slaughter has also been found (Huff *et al*., 1983).

Many negative effects of OTA on the immune system have been reported. Reduced lymphoid organs have been observed after feeding chickens with OTA, especially with regards to the bursa, as well as the spleen and thymus. Chang *et al*. (1979) found a reduced number of lymphocytes in chickens fed 0.5 ppm OTA and a reduced number of monocytes in chicken fed 2 ppm or more. Also, Campbell *et al*. (1983) found a reduced number of lymphocytes when they fed chickens 2 ppm OTA. They did not find impaired phagocytosis at 2 ppm, but Chang and Hamilton (1980) have reported it for chickens fed 4 ppm and more. Dwivedi and Burns (1984) found a

reduced number of immunoglobulins in sera from chickens fed 2 ppm OTA, which was the lowest dose tested. However, Campbell *et al*. (1983) could not find any effect on antibody titres in chickens fed the same OTA concentration.

Feeding OTA (≥0.5 ppm) contaminated feed to laying hens has been found to decrease egg production and feed consumption (e.g. Prior and Sisodia, 1978; Page *et al*., 1980). Results from a trial with restricted feeding showed that the decrease in egg production was probably not caused by the reduced feed consumption. Body weight gain and egg weight were also reduced from 1 ppm. The fertility and hatchability, on the other hand, were unaffected.

Turkeys are less sensitive to OTA than chickens. At OTA concentrations of 2 ppm in feed, the growth rate of the birds was depressed in a feeding study performed by Chang *et al*. (1981). At concentrations of 4 ppm the thymus weight was also lowered, plasma uric acid and water consumption was higher and the proventriculus and gizzard had increased in size.

Trichothecenes – cereals

DON is the most common trichothecene in poultry feed, originating mainly from wheat and maize ingredients. Both the acute and chronic toxicity of DON in poultry are relatively low when compared to the other trichothecenes.

Several feeding studies have been performed with graded DON from naturally or artificially infected cereals in the feed for broiler chickens. Hamilton *et al*. (1985b) fed wheat contaminated with 0.02–4.8 ppm DON to white leghorn and broiler chickens. They found no toxic effects. The same was found when Hulan and Proudfoot (1982) fed contaminated wheat in concentrations up to 1.87 ppm to chickens. In some trials with concentrations of 16–18 ppm, an increase in gizzard size, a decrease in growth rate and a decrease in feed efficiency have been found. Moran *et al*. (1982) undertook a study where they fed highly contaminated maize to chickens. When the concentrations were exceptionally high (≥116 ppm), they found growth inhibition and necrotic lesions in the mouth and gizzard of the birds.

Laying hens have also been fed different concentrations (0.7–83 ppm) of DON from naturally contaminated wheat in some trials (e.g. Farnworth *et al*., 1983; Hamilton *et al*., 1985a; Lun *et al*., 1986). In most of the studies no effect has been found on egg production, egg weight, shell thickness, weight gain and feed consumption. However, higher triglyceride content was found in the liver by both Farnworth *et al*. (1983) and Hamilton *et al*. (1985a) and small erosions in the gizzard were found by Lun *et al*. (1986) at the highest concentration (83 ppm). Decreased egg production has been found in many field cases where *Fusarium*-infected cereals or sorghum containing both DON and ZEA have been fed to laying hens.

In one study, turkey poults have been fed 2.2 and 4.4 ppm DON (Manley *et al*., 1988) and 5 ppm in another study (Hamilton *et al*., 1985b). No effects were found in terms of growth rate or feed consumption.

T-2 and HT-2 toxins are highly toxic for poultry, but levels resulting in acute lethal effects are uncommon. Poultry exposure to the toxins is mainly through oat, barley and maize ingredients.

Feeding experiments which gave graded T-2 toxin (0.2–16 ppm) to chicks have shown that the growth rate is initially retarded when they are fed more than 2 ppm (Chi *et al*., 1977b; Wyatt *et al*., 1973); however, oral lesions began to appear at 0.4 ppm. The appearance of oral lesions, which has been found in nearly all studies, seems to be an excellent indicator of T-2 intoxication. However, this may be influenced by the way in which the chickens are given water. Open water troughs, which can give the birds the opportunity to wash their beaks, may reduce oral lesions. The oral lesions first appear on the hard palate and along the margin of the tongue, then later large caseous discharges can be seen around the corner of the mouth and along the margin of the beak. These are raised lesions, yellowish-white in colour and caseous in texture. The plaques consist of fibrinous material, invaded by bacteria and granular leucocytes (Wyatt *et al*., 1972).

Feeding higher concentrations (≥4 ppm) of T-2 toxins to chickens has resulted in necrotic lesions appearing in the gizzard, abnormal feathering and neural disturbances with abnormal wing positioning and loss of the righting reflex. Hemorrhaging, which has often been associated with field fusario toxicosis, has not been found in any organs after feeding pure T-2 toxin to chicks. In feeding studies with chickens, T-2 toxins, which can inhibit lymphocyte production, have caused a reduction in the lymphoid organs, the bursa, the thymus and the spleen (e.g. Wyatt *et al*., 1973; Richard *et al*., 1978). The effect on the immune system has also been noted, as there was an increase in sensitivity to *Salmonella* infections (Boonchuvit *et al*., 1975).

Feeding T-2 toxins to laying hens for periods of eight or three weeks has shown that egg production, shell thickness and feed consumption were reduced in hens which were fed 8 ppm or more (Chi *et al*., 1977a; Speers *et al*., 1977). Oral lesions developed from 0.5 ppm of T-2 toxin in the feed, with erosions in the mucosa of the gizzard developing at higher concentrations. The hatchability of the eggs decreased when the hens were given 2 ppm T-2 toxin or more in their feed (Chi *et al*., 1977a).

Both body weight gain and thymus size were reduced when turkeys were fed between 2 and 10 ppm T-2 toxin for four weeks (Richard *et al*., 1978). Oral lesions also developed in the turkeys fed T-2 toxin.

Zearalenone – maize

Zearalenone (ZEA) is mostly known to cause estrogenic effects in pigs, but has very few acute, subacute or chronic effects in poultry. Feeding meals with high ZEA levels (≥300 ppm) to female broiler chickens resulted in an

increased growth rate, specifically in the areas of the comb, the ovary and the bursa (Speers *et al*., 1971). At higher concentrations fluid cysts were also found on the oviducts. For male chicks which were fed high ZEA concentrations, the growth rate of their combs decreased.

The effects of pure ZEA on laying hens are very low even at high concentrations (Allen *et al*., 1981a). The egg production was not reduced in any trials. However, decreased egg production has been seen in several field cases where both ZEA and DON have been found in the feed.

In turkeys fed 100 ppm ZEA, on the other hand, a moderate (20%) reduction in egg production has been found (Allen *et al*., 1981b). In male turkey poults ZEA has an androgenic effect, with dewlaps and caruncles developing earlier.

Fumonisins – maize

Poultry are relatively resistant to fumonisin toxicity; however, there is still a concern as there is a high use of maize in poultry diets. Day-old broiler chickens have been fed diets containing 0, 100, 200, 300 or 400 mg fumonisin B1 (FB1) from culture material per kg feed for 21 days (Ledoux *et al*., 1992). Body weight and daily weight gain decreased with FB1 concentration, whereas the weight of the liver, the proventriculus and the gizzard increased. Similar results were found in a second study, in which serum sphinganine levels and the serum sphinganine : sphingosine ratio increased at all doses (≥75 ppm FB1) (Weibking *et al*., 1993). Similarly, purified FB1 in the diet at 20–80 mg/kg increased the amount of sphinganine in the liver at all doses and in serum at 80 mg/kg (Henry *et al*., 2000). Hepatic serum enzymes and total liver lipids were elevated in the group fed the highest FB1 level.

Turkeys have also been fed diets containing 75–300 mg FB1 from culture material per kg feed for 21 days (Weibking *et al*., 1995). It was found that there was a dose-dependent decrease in feed intake and body-weight gain, together with an increase in the weight of the liver.

Feeding studies of Peking ducklings (Bermudez *et al*., 1995) and mallard ducks (Bailly *et al*., 2001; Tran *et al*., 2006) have shown that they are no more sensitive than broiler chickens.

11.4.3 Ruminants

Cereals, concentrates and silage fed to ruminants can often contain high amounts of diverse mycotoxins. Ruminants are generally considered to be less susceptible to the adverse effects of many mycotoxins. This assumption is based on the fact that the rumen flora will degrade and inactivate most mycotoxins. However, adverse effects to mycotoxins may be caused in ruminants by high toxin amounts, non-degradable mycotoxins and rumen disturbance. The role of mycotoxins in the health and performance of dairy cows has been reviewed by Fink-Gremmels (2008).

Aflatoxin – copra, cottonseed, maize gluten
Ruminants are exposed to aflatoxins mainly through feed components like peanut meal, cottonseed meal, copra, maize, maize gluten, rice and cassava. For this reason high amounts of aflatoxins may sometimes be consumed.

Direct toxic effects of aflatoxins in ruminants are mostly seen when they consume relatively high amounts. Feeding cows high amounts (>5 mg AFB1/day) of aflatoxin will result in lowered general health, reduced milk production and feed intake and, in more severe cases, liver damage and fatty liver. High-yielding dairy cows are considered more sensitive to AFB1 than fattening cattle (Applebaum *et al.*, 1982).

Rumen-degradable mycotoxins and disturbance – cereals, maize, silage
Several mycotoxins like OTA, ZEA, DON and other trichothecenes are degraded and mostly detoxified in the rumen. They may, however, cause toxic effects in young pre-ruminal animals. Adverse effects may also appear in ruminants where the rumen microbial flora and functions have been disturbed through special feeding regimes or feed with low hygiene levels. The disturbed ruminal functions can lead to decreased degradation of the mycotoxins and an increased absorption from the rumen and intestines.

A feed ratio with a high cereal portion will cause a low pH in the rumen. This low pH will reduce the number of rumen protozoa that are responsible for the main degradation of OTA (Kiessling *et al.*, 1984). Direct toxic effects of OTA have not been observed but a higher excretion into milk and contaminations of offal from slaughtered animals have been noted.

Ergot alkaloids – pasture grass, rye
There are several ergot alkaloids, the composition of which differs depending on type of fungus and infected plants. They are not inactivated by the rumen microflora, therefore the ruminants are susceptible to the alkaloids. There are several field cases of intoxications, but the type of ergot alkaloids and their concentration are seldom known.

Lameness and sometimes gangrene are common symptoms observed in cattle when the feed contains more than 10 g ergot/kg (Mantle, 1977). Pure ergotamine tartrate (1 mg/kg bw) was administered to six animals and four died within 10 days (Woods *et al.*, 1966). They became ill within 1–2 days with signs of anorexia, hyperventilation, cold extremities, salivation, and occasionally tongue necrosis.

In a dose–response study Holstein Friesian bulls were fed with three different doses (0, 0.45 and 2.2 g/kg concentrate) of ergot with a total alkaloid content of 633 mg/kg (Schumann *et al.*, 2007). No differences between the groups were found for live weight, feed intake, health conditions or liver enzymes and bilirubin in the blood. Higher amounts of alkaloids are probably needed to produce adverse effects.

Roquefortin C – silage
In silage consumed by ruminants and horses, roquefortin C frequently occurs in relatively high amounts. There are, however, very few reports of intoxications from roquefortin C. The toxin was, in one case, isolated from ensiled grains which had been fed to 34 dairy cows showing signs of paresis, indigestion, mastitis and abortion (Häggblom, 1990). The effects of roquefortin C have been investigated in sheep fed 0, 5 and 25 mg/kg silage over a period of 16 to 18 days (Tuller *et al*., 1998). No clinical signs of intoxication could be recognized during the experimental period. Neither serum biochemical and hematological parameters nor hormone profiles were changed by roquefortin. Therefore, it can be inferred that sheep seem to be insensitive to the toxin, but it is still unclear whether roquefortin can cause adverse effects in cows.

Fumonisins – maize
Maize, maize silage and maize screenings, which may contain fumonisins, are important parts of the diet for ruminants. Naturally occurring toxicosis has not been reported, but signs of liver toxicity and decreased feed intake and milk production have been noted in some cases (Richard *et al*., 1996; Baker and Rottinghaus, 1999). The fumonisins are not degraded in the rumen, or they are degraded only to a low degree (Gurung *et al*., 1999; Caloni *et al*., 2000). Experimentally, fumonisins are hepatotoxic and nephrotoxic to calves fed or intravenously administered high doses of fumonisins (Osweiler *et al*., 1993; Baker and Rottinghaus, 1999; Mathur *et al*., 2001).

11.4.4 Horses

The effect of mycotoxins in horses is mainly described in case studies such as outbreaks of mycotoxicoses and other clinical cases. Very few controlled feeding studies with mycotoxins have been undertaken in horses.

Fumonisins – maize
The most dramatic effects of mycotoxins in horses are those caused by fumonisins in maize and maize screenings. The fumonisins cause a syndrome called ELEM, equine leukoencephalomalacia, characterized by fatal necrotic lesions in the cerebrum. Neurological disturbances are seen in the movements of the horses, which are often going in circles. The fumonisins also induce cardiovascular dysfunction in horses with decreased heart rates, lower cardiac output, and right ventricular contractibility, which may be involved in the pathogenesis of the central nervous system (Smith *et al*., 2002).

Horses which were fed concentrates with fumonisin B1 at concentrations of 65, 130 and 200 mg/kg, respectively, from culture material from *Fusarium moniliforme*, for 27 days showed ELEM lesions at necroscopy (Goel *et al*., 1996). Evaluation of cases of ELEM in the USA indicate that the

consumption of feed containing more than 10 mg FB1/kg is associated with an increased risk of developing ELEM, whereas at concentrations <6 mg FB1/kg no increased risk is assumed (Constable *et al.*, 2000; Ross *et al.*, 1991).

Exposure of horses to fumonisins also induces liver and kidney damage together with an increase of free sphinganine in the serum. The increased sphinganine to sphingosine ratio precedes the signs of ELEM.

Trichothecenes (DON + HT-2/T-2) – oats

Horses can be highly exposed to trichothecenes (DON, T-2 and HT-2 toxins) through concentrates based on highly contaminated oats, barley or maize. There are, however, relatively few case reports on toxic effects caused by trichothecenes. Most cases are from Eastern Europe with the involvement of T-2 toxins and *Fusarium sporotrichioides*. The T-2 toxin has also been the cause of bean-hull poisoning of horses in Japan. Most of the trichothecene effects are therefore likely to be subclinical.

The effects of DON in horses have been studied in trials with highly (11–44 mg/kg) contaminated barley, maize or wheat, with the concentrates being fed to horses during a 40- or 21-day period (Johnson *et al.*, 1997; Raymond *et al.*, 2003, 2005). Feed intake, weight gain and blood clinical parameters were investigated in all trials and the athletic performance in one trial. The feed intake and weight gain were reduced in DON (~40 mg/day) fed horses compared to control, as well as in polymeric glucomannan fed horses in two trials. No reduction was observed in the consumption of the DON contaminated barley during the trial where the horses were fed approximately 50 mg DON/day through the barley concentrate. Serum γ-glutamyltransferase activities were higher in horses which were fed DON in one trial, but most other blood clinical parameters including serum IgM, IgG and IgA were not significantly affected. DON did not seem to affect the horses' athletic ability.

The exposure of 82 trotter horses to T-2 and HT-2 toxins through oats was studied in Sweden (Pettersson *et al.*, 2008). Horses consuming more than 1500 μg toxins per day were compared with those consuming less toxins. No clear effects of T-2 and HT-2 toxin consumption could be seen on health and performance of the horses.

Horses seem to tolerate rather high trichothecene concentrations in the concentrates, without showing clear adverse effects. The degradation of trichothecenes to the non-toxic de-epoxy trichothecenes in the intestinal content may contribute to the lack of effects.

Stachybotryotoxicosis – straw

Stachybotryotoxicosis is a mycotoxicosis mainly seen by its effects on horses. Severe outbreaks were reported from Eastern Europe during 1930–1950, but cases have also appeared in France more recently (Hintikka, 1977; Asquith, 1991). The toxic effects are caused by macrocyclic trichothecenes,

e.g. satratoxin produced by *Stachybotrys alternans* on feedstuffs, mainly straw consumed by the horses. The toxic signs are first recognized on eyes and mouth as swelling and inflammation of the mucosa, which later become necrotic. The number of leucocytes and trombocytes in the blood decrease and hemorrhages later occur in the mucous membranes, internal organs and muscles. Degenerative changes also appear in the liver, kidneys and myocardium. The horses have been known to die in the advanced stages of this intoxication. The toxin concentrations cannot usually be determined. Several macrocyclic trichothecenes may have been produced by the fungi. Stachybotryotoxicosis in horses is described in more detail by Hintikka (1977).

Aflatoxins – maize

Suspected cases of aflatoxin poisoning have been reported in horses consuming mouldy maize or hay (Angsubhakorn *et al*., 1981; Vesonder *et al*., 1991; Asquith, 1991). Clinical signs observed include CNS depression, anorexia, weight loss, icterus, and subcutaneous hemorrhages. During necroscopy, fatty livers, swollen kidneys, epicardial petechiae and hemorrhagic enteritis could be found.

Young ponies have been dosed with aflatoxin B1 (AFB1) in two studies (Aller *et al*., 1981; Bortell *et al*., 1983). In the first study the ponies were given 0.015 to 0.045 mg/kg bw as gastric intubations during 21 days, and then three months later they were given higher doses of 0.1–0.4 mg AFB1/kg bw for five days. The first treatment did not affect health, hematology or blood chemistry, but two horses given the highest dose in the second treatment showed increased activities of serum enzymes indicating hepatocellular toxicity. In the second study (Bortell *et al*., 1983) the ponies were given higher amounts (0.5–7.4 mg/kg bw) of AFB1 as a single oral dose. Ponies receiving AFB1 doses from 2 mg/kg bw died within 76 hours. The surviving ponies had elevated clinical blood enzymes indicating liver damage and increased protrombin time, hemoglobin and packed cell volume. The effects of AFB1 in horses are described in more detail by Asquith (1991).

Slaframine – forage

Slaframine toxicity in horses is recognized mainly by excessive salivation after they have consumed hay, silage or forage containing clover infected by *Rhizoctonia leguminicola*. The syndrome is called slobbers and intoxication of horses has mainly been reported in North America (Asquith, 1991) but has also recently occurred in Europe (Wijnberg *et al*., 2009). Clinical signs observed in the intoxication are excessive salivation, anorexia, diarrhea, polyuria, bloating, stiffness and even death. There are no feeding studies with known concentrations of slaframine and there is also lack of information on the concentration of slaframine in forage causing intoxications.

11.4.5 Fish and shrimps

The importance of mycotoxins in the farming of fish and shrimps has been elucidated through increased death and poor growth, when cereals, maize and oilseed by-products have been increased as feed components. The effects of mycotoxins in aquaculturing have been reviewed by Spring and Fegan (2005).

Aflatoxin – maize, rice

The hepatocarcinogenicity of aflatoxin B1 (AFB1) in rainbow trout is the most well-known effect of mycotoxins in fish. In the early studies during the 1960s, high amounts of AFB1 and aflatoxin mixtures were administered to trout, producing both acute lethal effects and hepatocarcinomas (reviewed by Lee *et al*., 1991). This trout model has later been used in many studies related to cancer development and potential interference from other compounds in the diet.

AFB1 is the most potent mycotoxin liver carcinogen in fish and the rainbow trout is especially sensitive. Feed levels as low as 0.4 ppb have resulted in liver cancer in 14% of the rainbow trout at an age of 15 months (Lee *et al*., 1968). A diet with 20 ppb AFB1 resulted in a cancer incidence of 56% at eight months and 83% at 12 months.

All salmonids are sensitive to AFB1 and liver cancer development, but there are clear differences in the sensitivity between different species as well as different strains. Other fish such as channel catfish can be relatively aflatoxin resistant and may not normally develop liver cancer (Manning *et al*., 2005a; Jantrarotai and Lovell, 1990). Early clinical signs of aflatoxin in fish, like decreased growth performance, anemia, liver and gastric necrosis, were only observed in catfish when levels of 10,000 ppb AFB1 were fed (Jantrarotai and Lovell, 1990). Another fish species thought to be moderately resistant to AFB1 is Nile tilapia. Feed levels of 2.5 ppm AFB1 per kg or more for eight weeks were needed to cause reduced growth rate and hematocrit (Tuan *et al*., 2002). Histological changes and necrosis of the livers were first noted at levels above 10 ppm.

Shrimps fed AFB1 contaminated feed may also show adverse effects. Histopathological changes of the hepatopancreas have been observed in *Penaeus* shrimps after both high (<50 ppm AFB1) and low (>50 ppb AFB1) feed levels (Lightner *et al*., 1982; Gopinath and Raj, 2009).

The toxicity and metabolism of AFB1 in aquacultured fish and shrimps are reviewed by Santacroce *et al*. (2008), which is a valuable reference for obtaining further information regarding the effects of aflatoxin.

Trichothecenes – cereals, maize

Deoxynivalenol (DON) has been fed to rainbow trout in only one study (Woodward *et al*., 1983). Feed refusal occurred when diets contained 20 mg/kg or more. Graded levels between 1.0 and 13 mg of DON per kg feed were also fed to the fish for four weeks and caused a progressively greater

decrease in the liveweight gains of the juvenile trout. Feed intake and feed conversion efficiency were also depressed. The DON feed levels used in the study were relatively high, but when the results are reviewed it can be claimed that rainbow trout are sensitive to DON (Spring and Fegan, 2005), and that catfish are more tolerant, since levels ≥15 ppm are required to negatively affect their performance.

Pacific white shrimps (*Litopenaeus vannamei*) were fed DON (0.2, 0.5 and 1 ppm) in their diet for up to 16 weeks (Trigo-Stockli *et al.*, 2000). Both body weight and growth rates were reduced in the shrimps on toxin diets. However, only growth rate was affected at the lowest level. The growth rates of black tiger shrimps (*Penaeus mondon*) fed 0.5, 1.0 and 2.0 ppm DON for up to eight weeks were not reduced in another study (Supamattaya *et al.*, 2005).

Graded levels of T-2 toxin (1, 2.5, 5, 10 and 15 mg/kg) in the diet have been fed to rainbow fingerlings for 16 weeks (Poston *et al.*, 1982). All levels from 1 mg/kg depressed growth rate, feed efficiency, hematocrit and hemoglobin. Mortality of the fish was increased in the groups fed 10 mg/kg or more.

No reduced growth rate could be seen in an earlier study, when lower levels of T-2 toxin (0.2 and 0.4 mg/kg) were fed to rainbow trout for 12 months (Marasas *et al.*, 1969). In fact the fish which were fed the toxin grew better than the controls, and no changes were found in the histopathological examination afterwards. T-2 toxin has also been fed to channel catfish in graded dietary concentrations of 0.625, 1.25, 2.5 and 5.0 mg/kg diet for eight weeks (Manning *et al.*, 2003b). Reduced weight gain, feed conversion, hematocrit and survivability were seen in the group fed the three highest toxin levels. Histopathological anomalies of the stomach, head and trunk kidneys were also observed in the three highest toxin groups. Catfish fed with T-2 toxin (1 or 2 mg/kg diet) were challenged by the bacterium *Edwardsiella ictaluri* and after 21 days showed 84–99% higher mortality rates than the control group (Manning *et al.*, 2005b).

Fumonisins – maize

The effect of fumonisins mainly from culture material have been studied in feeding trials with channel catfish, Nile tilapia and carp. Groups of catfish were fed diets containing fumonisin B1 (FB1) from culture material at 20, 80, 320 and 720 mg/kg during 10 weeks (Lumlertdacha *et al.*, 1995). Weight gain was significantly decreased by 15% and histopathological liver lesions were found in the group fed the lowest toxin level. Catfish have also been fed graded levels (0.7, 2.5, 5.0, 10.0, 20.0, 40.0 and 240 mg/kg diet) of FB1 for 12 weeks in another study (Li *et al.*, 1994). Decreased weight gain, feed consumption and feed conversion ratio, as well as histopathological changes, were found at levels ≥40 mg FB1/kg feed.

Groups of Nile tilapia have also been fed graded levels (10, 40, 70 and 150 mg/kg) of FB1 in their diet for eight weeks (Tuan *et al.*, 2003). Average weight gain was decreased at levels ≥40 mg.

Catfish and Nile tilapia therefore seem to be equal in sensitivity to FB1 and may tolerate levels up to 20 mg FB1/kg of feed. Carp (*Cyprinus carpio*) may, on the other hand, be more sensitive according to results from two different feeding studies. One-year-old carp were fed FB1 equal to 0.5 and 5.0 mg/kg body weight for 42 days (Pepeljnjak *et al*., 2003), which resulted in a loss of body weight and alterations of hematological and biochemical parameters, indicating liver and kidney damage in fish from both groups. Pathological alterations in the liver, endocrine and exocrine pancreas, kidney, heart and brain were detected in another study where the carp had been fed 10 mg FB1/kg diet for 42 days (Petrinec *et al*., 2004).

The effects of fumonisins have also been studied in white shrimps (*Litopenaeus vannamei*) and they seem to be even more sensitive. Histopathological changes in the hepatopancreas and enzyme activities have been seen in shrimps fed >0.5 mg FB1/kg in their diet (Mexia-Salazar *et al*., 2008; Burgos-Hernandez *et al*., 2005).

Ochratoxin – cereals

Acute toxicity of ochratoxin A (OTA) has been determined in rainbow trout (*Salmo gairdneri*) and sea bass (*Dicentrarchus labrax*). The LD50 of intraperitoneal administered OTA to rainbow trout was found to be 4.67 mg/kg of body weight (Doster *et al*., 1972) and the oral LC50 in sea bass was 277 μg/kg bw (El-Sayed *et al*., 2009).

Eventual hepatocarcinogenicity has been investigated in rainbow trout after embryo exposure of 5–25 ppm OTA or feeding 250 ppm OTA (Lee *et al*., 1991). No incidence of hepatocarcinomas was noted after 12 months.

A feeding study with graded levels (0.5, 1.0, 2.0, 4.0 and 8.0 mg/kg) of OTA in the diets of channel catfish for eight weeks has been performed (Manning *et al*., 2003a). Feed levels from 1.0 mg OTA/kg reduced weight gain, and higher OTA levels also reduced feed conversion efficiency and hematocrit values. Histological changes were only seen in the liver and kidney from fish which were fed the highest OTA level.

The effect of OTA has also been studied in Nile tilapia and African catfish (*Clarias gariepinus*) as reviewed by Spring and Fegan (2005). A reduction in the erythrocyte count, hemoglobin concentration and hematocrit in response to OTA were seen in both fish species at feed levels above 0.4 mg/kg.

11.4.6 Pets

Most information on the adverse effects of mycotoxins in pet animals is obtained from case reports on intoxications of animals. Major outbreaks of mycotoxicoses in pets between 1970 and 2006 have been reported in a review by Leung *et al*. (2006). The number of cases is highest for the dogs that were affected by aflatoxin. A total of 72 cases are reported and clinically described from the USA in 2005–2006 (Dereszynski *et al*., 2008).

The effects of mycotoxins in pets, especially dogs, are reviewed by Böhm *et al*. (2008).

Aflatoxin – maize

Dogs are highly susceptible to aflatoxin B1 (AFB1) in the feed and even commercial dog foods are in many cases contaminated with aflatoxins, mainly through maize.

In acute aflatoxicosis when dogs are exposed to >1 mg AFB1/kg of body weight they normally die within a few days showing enlarged livers, disseminated intravascular coagulation and internal hemorrhaging. Sub-acute aflatoxicosis (<0.5 mg AFB1/kg feed) is characterized by anorexia, lethargy, jaundice, intravascular coagulation and death within 2–3 weeks. Hepatotoxic effects can also be produced by chronic AFB1 exposure through 0.05–0.3 mg AFB1/kg feed over 6–8 weeks.

The clinical and clinicopathological features of AFB1 intoxications in dogs are thoroughly described by Dereszynski *et al*. (2008). The best early markers of aflatoxicosis in dogs were found to be low plasma activity of anticoagulant proteins (protein C, antirhombin) and hypocholesterolemia. Serum liver enzyme activities and bilirubin concentrations were unreliable early markers of AFB1 hepatotoxicosis in dogs.

Trichothecenes – cereals

Dogs are highly exposed to trichothecenes, especially deoxynivalenol (DON), through cereals and maize in dry extruded pet food. There are, however, relatively few case reports of clinical intoxications of dogs by DON.

Experimental feeding trials with DON in dogs and cats have been conducted to determine dietary amounts of DON that are required to produce overt signs of toxicity like vomiting and reduced feed intake (Hughes *et al*., 1999). Naturally contaminated wheat was used to produce extruded pet foods containing 0, 1, 2, 4, 6, 8 and 10 mg DON/kg, which were fed to groups of dogs or cats for a 14-day feeding period. Dog food intake was significantly reduced by DON concentrations greater than 4.5 mg/kg. Vomiting was observed at levels of 8 and 10 mg DON/kg food. Food intake by the cats was significantly reduced when the DON concentrations exceeded 7.7 mg/kg food. Vomiting was common at the highest concentrations of 8 and 10 mg DON/kg.

In a feeding trial on beagle dogs for 14 days, with a diet based on a blend of naturally contaminated grains containing mainly DON (4 ppm), polymeric glucomannans were added as a possible means to prevent the toxic effects caused by DON (Leung *et al*., 2007). The DON contaminated diet caused a reduction in feed intake and body weight. There was, however, no effect of the glucomannan addition.

Ochratoxin – cereals, offal, meat
Dogs are considered to be highly susceptible to ochratoxin A (OTA), but there are only a few feeding studies. A daily dose of 0.2 mg/kg of body weight for two weeks or a single dose of 7.8 mg OTA/kg of body weight was lethal to young beagle dogs (Szczech *et al*., 1973). Severe kidney lesions, which were located not only in the proximal tubules, could be observed. Clinical symptoms of OTA intoxication in dogs include anorexia, weight loss and vomiting to increased body temperature, dehydration and prostration (Boermans and Leung, 2007; Kitchen *et al*., 1977). Beagle dogs given OTA doses of 0.1 and 0.2 mg/kg of body weight for 14 days had visible kidney lesions with degeneration and necrosis of the proximal tubules (Kitchen *et al*., 1977).

OTA has been implicated in several reported cases of mycotoxin intoxications in dogs (Leung *et al*., 2006). OTA has also been detected in feline kidneys and pet foods for cats in Austria, but the concentrations were not considered high enough to have caused renal diseases in the cats (Pühringer *et al*., 2007).

Roquefortin and tremorgens – food leavings
There are several cases of dog intoxication with tremorgenic mycotoxins reported in the literature. Clinical signs reported are vomiting, convulsions, tremors, ataxia and tachycardia. Dogs have also died from this type of intoxication. The symptoms may in many cases have been confused with strychnine intoxication. Both penitrem A and roquefortin C have, however, been found in stomach contents and in food. These have most likely been produced by *Penicillium crustosum* on the food leftovers (e.g. rice, walnuts, apples, cheese, mouldy spaghetti) consumed by the dogs (Boysen *et al*., 2002). In some cases, especially with Roquefort cheese, only roquefortin C has been found. Dogs seem to be sensitive to these mycotoxins, but the exact concentrations have in most cases not been determined. The rice consumed in one case contained 2.6 μg/g of penitrem A and 34 μg/g roquefortin C (Naude *et al*., 2002).

11.5 Carry-over into food

11.5.1 Milk

The excretion and carry-over of mycotoxins into milk are of great concern, especially with regards to the carry-over of aflatoxin to milk, which has been investigated and risk-evaluated (EFSA, 2004). The carry-over of other mycotoxins into dairy milk has also been reviewed by Fink-Gremmels (2008).

Aflatoxin
The carry-over of aflatoxin to bovine milk is of great importance and is discussed in detail below. The major part of the aflatoxin B1 (AFB1)

consumed via feeding stuffs is degraded in the rumen of the dairy cows. A minor but important part is absorbed by the cow and rapidly metabolized into aflatoxin M1 (AFM1) in the liver. AFM1 is subsequently relatively stable and circulates in the blood until it is excreted in milk, urine or bile or is further metabolized.

Many studies have been performed on the carry-over of AFB1 from feeding stuffs to milk. In early studies (prior to 1985), as reviewed by Sieber and Blanc (1978), Applebaum *et al*. (1982) and Van Egmond (1989), quite variable results were obtained. The estimated carry-over of the consumed quantity of AFB1 to the quantity of AFM1 in the milk varied between 0.18% and 3.94% of the quantity consumed. In these early studies considerable quantities (in relation to the present maximum permitted level) of AFB1 were used for (single) low milk yielding dairy cows, and the chemical analysis methods for aflatoxin were often of low quality. This may explain the great variation, and the results must be deemed unreliable.

Since 1985, nine reports have been published (Price *et al*., 1985; Frobish *et al*., 1986; Fremy *et al*., 1987; Munksgaard *et al*., 1987; Pettersson *et al*., 1990; Harvey *et al*., 1991; Veldman *et al*., 1992; Veldman, 1992; Galvano *et al*., 1996) and some are very accurate investigations, extensive, and using quantities around the maximum permitted level. These more recent studies also show variations of between 0.32 and 6.2% in the estimated carry-over of AFB1. The mean value for all reports is 1.81% with a standard deviation of 1.22. If data is used exclusively from cows that have been fed a maximum of 500 μg of AFB1 per day, a carry-over of 1.63% is obtained.

This considerable variation is due to a number of reasons. Apart from variation as a result of analytical problems and experimental techniques, there are also considerable individual excretion differences. This individual variation has been pointed out in several of the studies, but it is outweighed to a certain extent by the fact that the studies have been performed on groups of cows. Differences dependent on breed may be present, but this is not clearly discernible in the studies available. The cows' production levels are also of importance to the indicated variation. The Swedish study (Pettersson *et al*., 1990) and the Dutch study (Veldman *et al*., 1992; Veldman, 1992), with high milk producing dairy cows, revealed the highest carry-over: 2.6% and 2.7–6.2% respectively. Veldman *et al*. (1992) demonstrated clearly that carry-over is increased in relation to increased milk production and if the data from all the studies is analysed an increase of approximately 0.1% per kg of milk occurs. The mean carry-over value in high milk producing cows (>25 kg/day) is 2.66 ± l.24%. The major part of this carry-over is a result of the increased milk production and the resulting higher quantity of AFMl excreted.

A better and more natural linear relationship seems to exist between the quantity of AFB1 consumed per day and the concentration of AFM1 in the milk. This relationship is also less dependent on milk production. If all the data are analysed by regression, the outcome is that the AFMl

concentration in the milk is increased by 0.72 ng for every μg of AFBl consumed, with a correlation coefficient of 0.943.

Higher milk producing cows have a higher excretion rate of AFM1. This is assumed to be the result of a higher permeability in the cell membranes of the alveoli (Veldman *et al*., 1992). Cows afflicted by mastitis also have a higher excretion of AFM1, which is believed to be the result of an increased permeability of the membranes (Veldman *et al*., 1992). Feeding and milking routines may also influence the carry-over, but have not been studied.

Ochratoxin A

Transmission of ochratoxin A (OTA) into milk has been shown in monogastric animals, but has not been studied in ruminants. OTA is rapidly degraded in the rumen, to non-toxic ochratoxin alpha. Some OTA may escape rumen metabolism and be absorbed, as it has been found in low concentrations in bovine milk from Sweden and Norway (Breitholtz-Emanuelsson, 1993; Skaug, 1999). It was, however, not found in German retail milk from a survey in 1996 (Valenta and Goll, 1996). The concentrations of OTA found in the milk were relatively low (10–58 ng/L), but the total intake by high consumers such as children may be of high concern.

Fusarium toxins

Several studies with ZEA to dairy cows have shown a low transmission into milk and only trace amounts of the toxin and its metabolites have been found (Prelusky *et al*., 1990; Usleber *et al*., 1992; Goll *et al*., 1995; Seeling *et al*., 2005). Surveys of ZEA in retail milk samples have also shown low levels of contamination (EC, 2003).

The carry-over of DON and its metabolites into bovine milk has been investigated in some studies (Prelusky *et al*., 1984; Cote *et al*., 1986; Charmley *et al*., 1993; Seeling *et al*., 2006; Keese *et al*., 2008). No, or very low, transmission of DON and metabolites were found under normal feeding conditions.

Transmission of T-2 toxin into bovine milk was investigated in an early study by Robison *et al*. (1979). With a high feed level of 50 mg/kg they found 10–160 μg/kg in the milk.

The carry-over of FB1 has been found to be very low or absent in feeding studies on dairy cows (Prelusky, 1994; Richard *et al*., 1996).

11.5.2 Meat and offal

Most information on the carry-over of mycotoxins to meat and offal is obtained from residue analyses after feeding studies with mycotoxins to different animals. Residue analyses of mycotoxins in retail animal food products have also added further information.

Aflatoxin

Residues of aflatoxins in muscle/meat were investigated after many early feeding studies with pigs, cattle and chickens before 1980 (Rodricks and Stoloff, 1977). Only traces (0.01–0.5 μg/kg) of AFB1 and M1 at or just above the detection limits of the analytical methods were found in the muscle tissues from pigs in some feeding trials (Jacobson *et al*., 1978; Furtado *et al*., 1979). The aflatoxins were not detected in muscles from cattle when fed even relatively high amounts (1250 ppb) of AFB1 (Hayes *et al*., 1977; Helferich *et al*., 1986). In some studies where broiler chickens have been fed high concentrations (1–5 ppm) of AFB1, relatively high residues (5–26 μg/kg) of the aflatoxins have also been found in muscle tissues (Mintzlaff *et al*., 1974; Pandey and Chauhan, 2007). Other studies with similarly high AFB1 feed levels to broilers have found only low levels (<0.2 μg/kg) of aflatoxins in the muscle tissues (Chen *et al*., 1984; Wolzak *et al*., 1986; Fernandez *et al*., 1994; Bintvihok *et al*., 2002). The leg muscle seems to often have a slightly higher contamination than the breast muscle.

Ochratoxin A

In a number of feeding experiments in Denmark and Germany, residues of ochratoxin A (OTA) in tissues and blood from pigs have been studied (Krogh *et al*., 1974; Madsen *et al*., 1982a, b; Mortensen *et al*., 1983a, b; Lusky *et al*., 1993, 1994, 1995). In most cases the feeding has been executed using naturally contaminated feeding stuffs. Levels of OTA in the feed have been between 25 and 1989 μg per kg. All studies showed that residue concentrations in slaughtered pigs can be ranked as follows: serum levels > kidney > liver > muscle tissue and fat. Muscle tissues are thus less contaminated. In pigs fed between 1 and 2 ppm OTA, the residual concentration in the kidney was 14–67 ng/g, in the liver it was 10–30 ng/g and in the muscle it was 4–44 ng/g (Madsen *et al*., 1982a).

After feeding chickens diets containing various OTA levels, residue analyses have been performed by Krogh *et al*. (1976), Prior *et al*. (1980), Golinski *et al*. (1983), Niemiec *et al*. (1988) and Micco *et al*. (1987, 1988). Most residue analyses have been performed after feeding with more than 1000 μg OTA per kg of feeding stuffs. The residues found in the liver and muscles were in the range of 0.4–22 and 0.1–0.62 ng/g respectively. In only two studies (Micco *et al*., 1987; Krogh *et al*., 1976) the levels of OTA in feeding stuffs neared the maximum permitted. In these experiments, the residues in the liver were 2 and 11 ng/g respectively and in the muscles 2 ng/g were observed.

Fusarium toxins

Residue analyses of DON, and its metabolite DOM, in muscle tissues from pigs fed DON concentrations ranging between 0.5 and 6.7 ppm DON revealed in most cases low (<20 ng/g) or undetectable DON concentrations

(Pollmann *et al*., 1985; Cote *et al*., 1986; Lusky *et al*., 1998; Schneweis *et al*., 2005; Goyarts *et al*., 2007; Döll *et al*., 2008). There is no clear relation between residue concentrations and the level of DON in feed, although the highest residue concentration (14 ng/g) was found after feeding the animals 6.7 ppm of DON.

DON residues have not been found in either breast or leg muscles from chickens, even when fed with high concentrations of DON (El-Banna *et al*., 1983; Kubena *et al*., 1985; Dänicke *et al*., 2007). Neither DON nor DOM could be found in muscle tissues from broilers fed 2.5 ppm DON, even when a method was used which could detect levels at 1.5 ng/g in fresh tissue (Dänicke *et al*., 2007).

Residues of NIV have not been found in the liver or muscles, but have been found in bile from pigs fed 5 ppm NIV (Hedman *et al*., 1997). In hens fed 5 ppm NIV, traces of NIV have been found in the liver and bile, in amounts up to 11 ng/g (Garaleviciene *et al*., 2002).

Neither T-2 toxin nor diacetoxyscirpenol has been found as a tissue residue, using analytical chemistry methods. This is probably due to the fact that they are rapidly metabolized, at least to their deacetylated metabolites. Small amounts of diacetoxyscirpenol (22 ng/ml) and its metabolites (13 and 15 ng/ml) have been found in the serum of pigs fed 2 ppm diacetoxyscirpenol (Bauer *et al*., 1985).

There are some studies that have assessed the carry-over of ZEA into edible tissues of pigs (Goyarts *et al*., 2007; James and Smith, 1982; Zöllner *et al*., 2002; Döll *et al*., 2003). In pigs fed 0.01–0.7 mg ZEA/kg diets, low levels of ZEA have been detected primarily in the liver (0.1–12 ng/g). Traces of ZEA and up to 14.5 ng/g of α-ZOL were found in muscle tissue from one study.

After feeding laying hens 1.1 ppm ZEA, residues of ZEA (<1–3.2 ng/g) and α-ZOL (3.5–3.8 ng/g) could be found in the liver but no residues were found in the breast muscle (Dänicke *et al*., 2002). High amounts (170–416 ng/g) of ZEA were, however, found in the kidney, liver and muscle from chickens given 10 mg ZEA per kg bw (Maryamma *et al*., 1992).

Radioactively marked fumonisin has been used to assess the carry-over into tissues of pigs and laying hens dosed with 2–3 mg fumonisin/kg bw (Prelusky, 1994; Prelusky *et al*., 1996). Fumonisin was found in the liver and kidneys from both pigs and laying hens, at levels in pigs of up to 160 ng/g, and in laying hens of 530 ng/g. Other tissues including muscle had low levels (<10 ng/g). In a more recent study by Meyer *et al*. (2003) pigs were given high doses (50 and 100 mg FB1/day) for 5–11 days, and residues of FB1 could be detected in the kidneys (mean 833 ng/g), liver (231 ng/g), lung (170 ng/g), spleen (854 ng/g), muscle (26 ng/g) and fat (2 ng/g) (Meyer *et al*., 2003). Some exceptionally high residue concentrations were also found in individual animals (kidney 4760 ng/g, spleen 7980 ng/g, liver 710 ng/g, lung 1150 ng/g and myocardium 838 ng/g). These studies concluded that

feeding pigs a ration containing 2 mg fumonisin B1 per kg would lead to residues of importance for human exposure.

11.5.3 Eggs

Laying hens can tolerate rather high levels of mycotoxins without clear and drastic toxic symptoms. They may therefore have relatively high mycotoxin levels in the blood during egg production, meaning that there will be a high risk of contamination of the produced eggs.

The aflatoxin residues in eggs from hens fed high (0.4–5 ppm) AFB1 levels have mostly been below 1 μg/kg (Lotzsch and Leistner, 1977; Fernandez *et al*., 1994; Oliveira *et al*., 2000; Zaghini *et al*., 2005), but in three studies higher residues (1.4–5.8 μg/kg) have been found (Trucksess *et al*., 1983; Bintvihok *et al*., 2002; Pandey and Chauhan, 2007).

OTA was found in eggs, both egg white and yolk, after high levels (>1.3 ppm) were given to the laying hens through their feed (Juszkiewicz *et al*., 1982; Bauer *et al*., 1988; Niemiec *et al*., 1994). The toxin is mainly found in the yolk, at concentrations of between 1.3 and 7.9 ng/g.

Zearlenone transmission into eggs is low or absent in most studies. Administration of ZEA as a radioactive single dose of 10 mg/kg bw resulted in radioactivity equal to 2 μg ZEA equivalents per gram in the yolk of eggs produced after 72 h (Dailey *et al*., 1980). This represents both ZEA and its metabolites. In two later studies both ZEA and its metabolites have been chemically analysed in eggs from hens fed 0.5 or 1.1 mg ZEA/kg (Dänicke *et al*., 2002; Sypecka *et al*., 2004). Neither ZEA nor its metabolites α- and β-zearalenol could be found above the detection levels of 0.1–7 ng/g. It was concluded that eventual transmission into eggs ought to be less than 0.03% of the feed level.

In a survey of mycotoxins in Belgian eggs, however, ZEA and its metabolites were detected in 12 of 20 analysed combined egg samples (Tangni *et al*., 2009). They were found at trace levels, above the detection levels of 3–6 ng/g.

Traces of DON and the metabolite de-epoxy-DON may be transferred to eggs if the hens are fed high amounts (>5 ppm) of DON in their feed. Valenta and Dänicke (2005) could not find DON or DOM in eggs from hens fed 11.5 mg DON/kg when using an analytical method with a detection of 2.5 ng/g in yolk and 1.0 ng/g in albumen. A LC-MS method with lower detection (0.01 ng/g) was used by Sypecka *et al*. (2004) for analysing DON in eggs from hens fed either 5, 7.5 or 10 mg DON/kg feed. DON was detected in nearly all eggs at low levels between 0.19 and 0.63 ng/g. There were no clear differences in the residue levels between the groups fed the three different levels of DON.

DON and DOM were also detected in the Belgian survey of home-produced eggs (Tangni *et al*., 2009). DON was detected in 17 of the 20 combined egg samples and DOM in only four. The median concentrations

of DON for autumn and spring egg samples were 2 and 3 ng/g respectively.

Tangni *et al*. (2009) have also made intake calculations and risk exposure assessments of mycotoxin (DON, ZEA, OTA, citrinin) residues in home-produced eggs for Belgian consumers. Weekly DON intake was calculated to be only 0.02–0.84% of the PMTWI. It was concluded that the mycotoxin intake from eggs is of no concern in relation to the tolerable daily intake.

11.6 Detection methods

Several analytical methods have been developed for analysis of the most common mycotoxins aflatoxins, OTA, ZEA, fumonisins and DON in cereals and food. Some are also adjusted to include analysis in mixed feeds (for details see Chapter 12).

11.7 Methods for the prevention of mycotoxin

11.7.1 Good agricultural practice

Many mycotoxins are already formed in the crop during growth in the field. The degree of fungal infection and toxin production is dependent on several culturing conditions. Previous crop, soil cultivation, variety selection, sowing time, weather conditions, fungicide use, harvest time, preservation and storage conditions are all important agricultural factors which can be controlled and adjusted to avoid fungal growth and toxin production. Good agricultural practice can be used to reduce the risks. Codes of good agricultural practice to reduce mycotoxins in cereals have been produced by CODEX, EU and different nations (CODEX, 2003; EC, 2006b; FSA, 2007a). They deal mainly with recommendations on how to reduce the fusarium toxins DON and ZEA. There are also special recommendations for the reduction of OTA (CODEX, 2003; FSA, 2007b). The recommended practice will, however, not reduce all mycotoxins and some may even increase due to recommended practice.

11.7.2 Hazard analysis and critical control point (HACCP) and good manufacturing practice (GMP) at feed mills

For feed production in feed mills HACCP and GMP should be used to reduce the risks of mycotoxin contamination. The use of GMP and HACCP in feed quality assurance is described in more detail in Part VI of this volume.

General quality control and choice of feed ingredients are the first steps. Control analysis for certain mycotoxins in the ingredients may sometimes be a control point, especially for materials with a high risk of mycotoxin

contamination. Purchase certificates on the absence of mycotoxins in bulk feed materials are more often required, thus forcing control analyses to be undertaken in the first stage of production. This control is especially important in the EU for AFB1 in dairy feed components. Analysis can be done of some selected mycotoxins in the finished compound feed, but this is more commonly done as random sampling.

11.7.3 Rapid alert

When the national controlling authorities within the European Union discover a feed or food consignment with a mycotoxin level above the maximal permitted level, they will notify the Commission. These notifications are published on the Internet in the Rapid Alert System for Food and Feed (RASFF), which has been in use for 30 years now. Other controlling authorities, food factories and feed mills will be warned by RASFF about consignments of feed and food materials which may contain mycotoxin levels above permitted levels. They can then prevent such material entering their feed products and the food chain within the Union.

11.7.4 Feed additives

Under the current conditions of agricultural practice, feed contamination with mycotoxins seems unavoidable. Various attempts have been made to prevent absorption and/or to reduce the detrimental effects of mycotoxins at the farm level. The strategies and products are discussed in Chapter 13.

A scientific review of mycotoxin-detoxifying agents used as feed additives was also requested by EFSA and has recently been published on the Internet (http://www.efsa.europa.eu/en/supporting/doc/22e.pdf).

11.8 Regulatory control

Most countries have nowadays introduced at least some regulations for mycotoxins in food and feed in order to reduce human and animal exposure. The situation and extent of regulation in all countries in the world in 2003 were presented and summarized by the FAO (FAO, 2004). Most regulations are for food products, with aflatoxins being the most commonly regulated mycotoxin. The regulations for feed are also mainly for aflatoxins, but the type of feed material and the animals it is intended for are also specified. The maximum limits for commonly regulated mycotoxins in animal feeds are presented in Table 11.4 for the EU, USA, Canada, Iran, Ukraine, and other countries which have regulated more mycotoxins than just aflatoxins. The maximum limits vary a lot between countries for the same mycotoxin and feed. The rationales behind setting the limits must have been different. The types of statutory limits are not always clear with

Table 11.4 Maximal and advisory levels of mycotoxin in feeds in different countries

Toxin	Animal/feed	EU	USA	Canada	Japan	China	Ukraine	Iran
Aflatoxin	Dairy, complementary	0.005	0.020	0.020	0.010	0.010	0.050	0.005
	Cattle, mature	0.050	0.020	0.020	0.020	0.020		0.050
	Pigs	0.020	0.100	0.020	0.020	0.020	0.050	
	Pigs, complementary	0.030						
	Poultry	0.020	0.100	0.020	0.020	0.010	0.025	0.010
	Finishing beef cattle	0.050	0.200	0.020	0.020	0.050	0.100	0.050
Deoxynivalenol	Feed cereals	8	5/10					
	Maize by-products	12						
	Pigs	0.9	5	1	0.200	0.500	1	
	Sows	0.9	5	1	0.200	0.500	1	
	Cattle	5	10	5	4	0.500	1	5
	Calves	2	5	1	4	0.500	1	1
	Poultry	5	10	5	1	0.500	1	
Fumonisins	Horses	5	5					
	Pigs	5	20					
	Poultry	20	100					
	Cattle	50	60					
Ochratoxin A	Feed cereals	0.25						
	Pigs	0.05		2		0.020		
	Poultry	0.1		2		0.020		
	Cattle					0.020		
Zearalenone	Feed cereals	2						
	Maize by-products	3						
	Pigs	0.25		3	0.020	0.100	2	
	Sows	0.1		3	0.020	0.100	0.040	
	Poultry				1	0.100		
	Cattle	0.5			1	0.100		
T-2 + HT-2	Pigs			1		0.080		
	Poultry			1		0.080	0.200	
	Cattle					0.080	0.250	0.100
	Dairy cattle					0.080		0.025

regards to the maximum limit or a recommended maximum. Since the 1970s, AFB1 and rye ergot have been regulated as undesirable substances in feed in the EU. The limits for AFB1 and rye ergot are given in Table 11.5, as presented in the Directive 2002/32/EC (EC, 2002).

The EU limits for mycotoxins other than aflatoxins are only guidance values. They are low compared to the levels set in other countries, but have been established in line with a toxicological risk assessment conducted by the CONTAM Panel of the EFSA. These guidance values for DON, ZEA, OTA and fumonisins were introduced in 2006 by Commission Recommendation 2006/576/EC (EC, 2006a) and they are also completely presented in Table 11.5. T-2 and HT-2 toxins are mentioned in the Commission Recommendation but no guidance values are set as yet. Upon request of the European Commission, in 2011 the CONTAM Panel of EFSA started to issue Opinions about other mycotoxins, including ZEA and all major *Fusarium* toxins (T-2, HT-2, NIV, etc.), *Aspergillus* toxins and *Alternaria* toxins. These EFSA Opinions will form the basis for further recommendations about maximal tolerance levels for mycotoxins in food and feed materials.

These recommendations for maximum limits and guidance values are primarily set for use by feed business operators to judge the acceptability of compound feed and cereal, and cereal products for animal feeding. They are suggested by the EC to be included as critical control points in the feed companies' HACCP systems. These are probably not fully introduced in all companies, due to the complicated and costly analysis of critical control points. For some feeds, e.g. dairy complementary feed, it is important that a stricter or lower limit for AFB1 is used. This is a response to the market, which demands the absence of AFM1 from milk and dairy products.

11.9 Future trends

New regulations for additional mycotoxins in feed will appear in many countries in the next few years. The present limits will be reconsidered and some levels decreased. There will be a higher degree of consensus and harmonization of the regulations as has already started with the acceptable mycotoxin concentrations in food.

Control, both official and by the feed industry, will increase in order to improve the quality for marketed feeds. The quality and reliability of rapid (real-time) methods for the analysis of mycotoxin, which can be used in the feed companies, will improve, whilst the costs for these ready-to-use kits will decrease. New methods for the recently regulated mycotoxins will be standardized for use in official controls. Multi-mycotoxin methods based on LC-MS/MS will be validated for feed survey purposes and adjusted for reliable quantification of mycotoxins in various feed matrices. The use of multi-mycotoxin methods based on LC-MS/MS will

Table 11.5 Guidance values for mycotoxins in animal feeds within the European Union

Mycotoxin	Products intended for animal feed	Guidance value (mg/kg)
Deoxynivalenol	Feed materials	
	– Cereals and cereal products with the exception of maize by-products	8
	– Maize by-products	12
	Complementary and complete feedingstuffs with the exception of:	5
	– complementary and complete feedingstuffs for pigs	0.9
	– complementary and complete feedingstuffs for calves (<4 months), lambs and kids	2
Zearalenone	Feed materials	
	– Cereals and cereal products with the exception of maize by-products	2
	– Maize by-products	3
	Complementary and complete feedingstuffs	
	– complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
	– complementary and complete feedingstuffs for sows and fattening pigs	0.25
	– complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lambs) and goats (including kids)	0.5
Ochratoxin A	Feed materials	
	– Cereals and cereal products	0.25
	Complementary and complete feedingstuffs	
	– complementary and complete feedingstuffs for pigs	0.05
	– complementary and complete feedingstuffs for poultry	0.1
Fumonisin B1 + B2	Feed materials	
	– maize and maize products	60
	Complementary and complete feedingstuffs for:	
	– pigs, horse (Equidae), rabbits and pet animals	5
	– fish	10
	– poultry, calves (<4 months), lambs and kids	20
	– adult ruminants (<4 months) and mink	50

also identify new or uncommon mycotoxins as potential contaminants of animal feeds. Feed additives such as mycotoxin-detoxifying agents are now approved for this purpose within the EU, but additional feeding studies are needed to prove the claimed effectiveness of these additives in daily practice.

11.10 References and further reading

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12

Detection and determination of natural toxins (mycotoxins and plant toxins) in feed

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Abstract: Animal feed is often contaminated with different types of mycotoxins and plant toxins. These toxic substances can cause adverse effects on animal health, and carry-over of some toxins and/or their metabolites into edible tissues, milk and eggs may contribute to human exposure. Therefore risk assessments and official controls of feed samples have to be performed. In order to carry out these assignments, different tools such as rapid screening tests and chromatographic confirmatory tests have been developed to analyse and detect the toxic substances in different commodities. An overview of the detection and determination of mycotoxins and plant toxins in feed is presented.

Key words: feed analysis, mycotoxin, plant toxin.

12.1 Introduction

Natural toxins are harmful organic compounds of natural origin that can occur in food and feed products causing acute or chronic toxic effects. On the basis of their origin the natural toxins can be divided into five main categories, namely mycotoxins, bacterial toxins, phycotoxins, plant toxins and zootoxins. Mycotoxins are toxins produced by fungi, while bacterial toxins are produced by bacteria. Phycotoxins are produced by algae and end up in fishery products through the food chain. Plant toxins are produced by edible plant species and zootoxins are produced by animals. The plant toxins and the zootoxins are inherent components of plants or animals. Because mycotoxins, bacterial toxins and phycotoxins can be produced by microorganisms, these toxins are classified as bio-contaminants (van Egmond, 2004). Mycotoxins and plant toxins are the most important

occurring natural toxins in animal feed; therefore this chapter will be restricted to these types of toxins.

12.2 Detection and determination of mycotoxins

12.2.1 Introduction

Mycotoxins are secondary fungal metabolites with toxic effects for humans and animals. The toxic effect of mycotoxins on animal and human health is referred to as *mycotoxicosis*, the severity of which depends on the intrinsic toxicity of the mycotoxin, the extent of exposure, age and nutritional status of the individual and possible synergistic effects with other chemicals to which the individual is exposed (Peraica *et al*., 1999) (for details about animal health effects see Chapter 11). Moulds of the genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* belong to the most important mycotoxin producers. *Aspergillus* and *Penicillium* species are generally found as contaminants in crops during drying and storage, so are also called the 'storage fungi', whereas *Fusarium* and *Alternaria* species are called the 'field fungi' because of the production of mycotoxins before or immediately after harvesting (Zollner and Mayer-Helm, 2006). As for fungal growth, many different factors can influence mycotoxin production, such as temperature, substrate aeration, water activity, inoculum size, microbial interactions, irregular storage and transport conditions (Garcia *et al*., 2009; Logrieco *et al*., 2003). Measurement of the actual toxin concentration in feed or food is an essential part of exposure assessment and overall hazard characterization. Subsequently, analytical tools have been developed and validated for different kinds of crops and commodities that allow monitoring of mycotoxin occurrence and the control of compliance with established statutory levels for mycotoxins in feed and food.

12.2.2 Sampling

In general, analytical testing can be divided into different consecutive steps. During the first step a selection of a representative sample of a given size from a bulk lot is made (the statistics applied in determining sample sizes are illustrated in Chapter 23). The sampling step is followed by the sample preparation step in which the grinding process and the sub-sampling take place. Finally, in the analytical phase several steps are executed, such as the extraction of the mycotoxins from the matrix, the clean-up of the extract and the detection of the mycotoxins in the purified extract. Because of the heterogeneous distribution, including the occurrence of 'hot spots' of these contaminants in feed commodities, the sampling step usually is the largest source of error and therefore affects the reliability of the measured levels

of mycotoxins. A statistically correct sampling plan that covers the whole lot is a crucial requirement. A large number of incremental samples joined together will form the overall aggregate sample representative of the lot. Appropriate sampling procedures will reduce the variability of the results and the number of misclassified lots. A false negative result implies that a non-compliant lot was put on the market affecting the risk to consumers, while a false positive result or a harmless withdrawal presents a financial burden for the producer (Brera and Miraglia, 1996; Brera *et al*., 1998; Cigic and Prosen, 2009; Miraglia *et al*., 2005; Stroka *et al*., 2004b; Whitaker, 2003). Commission Regulation 2006/401/EC lays down methods of sampling for official control of the levels of mycotoxins in foodstuffs. In this legislation a sampling method for cereal and cereal products is described. Until now no official regulations were established for feed commodities. A lot will be accepted or rejected if the laboratory sample is compliant to or exceeds the maximum limit, taking into account the corrections for recovery and measurement uncertainty.

12.2.3 Principles of determination and detection

After sampling, the sample is ground to reduce the size and to homogenize the sample during the sample preparation. In this way a representative subsample can be obtained for further analysis. For the analytical step two approaches can be distinguished, namely screening tests and confirmatory tests. Rapid screening tests allow mycotoxin determination in a non-laboratory environment and are mainly based on immunochemical techniques and various non-immunochemical techniques. These tests are simple, easy to use and cost-effective. However, in order to avoid misinterpretations, positive results need to be confirmed with more selective methods. Confirmatory tests are sensitive and selective chromatographic methods, with the most important drawbacks being the time-consuming sample extraction and clean-up step, the need for specially trained personnel and the more expensive equipment. Thin-layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled to different types of detectors such as ultraviolet, fluorescence and mass spectrometry (MS) are the most important confirmatory methods (Monbaliu *et al*., 2007).

In the first step during the analytical procedure a suitable extraction solvent is necessary to extract the mycotoxins out of the feed matrix. The extraction solvent, the extraction time and the equipment are important parameters in obtaining a good extraction efficiency. Unless the crude extract is used for analysis, liquid–liquid extraction, solid-phase extraction (SPE) and combined procedures are commonly used clean-up techniques for feed analysis (Cigic and Prosen, 2009; Krska *et al*., 2008; Prieto-Simon *et al*., 2007).

12.2.4 Applications

Screening methods

The early detection of mycotoxin-contaminated agricultural commodities is essential so that contaminated ingredients can be rejected or diverted either for human food or for animal feeding. This need has led to the development of reliable screening methods and has resulted in two types of application methods, namely field tests which are easy to perform and to interpret by users who are close to the site of contamination, and non-field tests which require a detection instrument. The immunochemical methods occupy a leading place in the screening group of methods for the determination of mycotoxins. The specific interaction between a mycotoxin and the respective antibody has led to the successful development of sensitive and specific mycotoxin immunoassays for feed analysis. Besides these benefits, the use of antibodies implies certain limitations such as the need to use animals for its initial production and the susceptibility to denaturation or proteolytic degradation. The stimulation of the animal's immune system results in the production of antibodies in an aqueous phase, but the use of organic solvents for mycotoxin extraction can affect the mycotoxin affinity and the antibody stability. Although the antigen–antibody interaction is a very specific interaction, this technique is susceptible to cross-reactivity caused by structurally related compounds. Cross-reactivity and other conditions such as temperature, pH and matrix effects can result in a significant overestimation of mycotoxin concentration (Cigic and Prosen, 2009; Goryacheva *et al*., 2009; Krska *et al*., 2008; Prieto-Simon *et al*., 2007; Reiter *et al*., 2009).

The immunodiagnostic assay is based on a direct or indirect competition (Fig. 12.1). In the direct competition the analyte (antigen) and an enzyme-labelled antigen compete for binding with antibodies which are immobilized on a solid support. The indirect competition involves an antigen conjugated with a macromolecular support bound to a solid phase followed by the addition of specific antibodies and analyte. Anti-species antibodies labelled with an enzyme are added to determine the amount of bound antibodies (Goryacheva *et al*., 2009).

Different types of immunochemical methods (Fig. 12.2) have been developed, such as the classic enzyme linked immunosorbent assay (ELISA) using a multi-well microtitre plate with visual, spectrophotometric or electrochemical detection. In the non-instrumental flow-through enzyme immunoassay, or immunofiltration assay, antibodies are immobilized on a semi-permeable membrane. A direct competitive immunoassay is further performed while colour production is visually evaluated. The lateral flow immunoassay or immunostrip or dipstick uses a test strip which is composed of a conjugate pad, a membrane, an absorbent pad and an adhesive backing. The detector reagent, typically an antibody coupled to latex or a colloidal particle, is deposited into the conjugate pad. When the sample is added to the conjugate pad, the detector reagent is solubilized and starts migrating

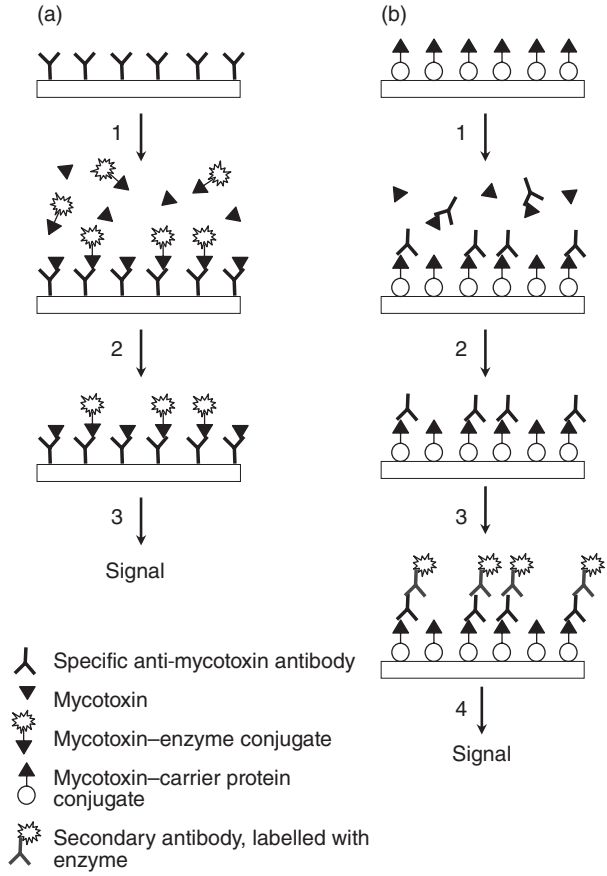

Fig. 12.1 Principles of (a) direct and (b) indirect competition of an immunodiagnostic assay. (a) 1 – Application of sample with addition of mycotoxin–enzyme conjugate, 2 – washing step, 3 – substrate application. (b) 1– Application of sample with addition of specific antibody, 2 – washing step, 3 – application of secondary antibody labelled with enzyme, 4 – substrate application. From Goryacheva *et al*., 2009.

with the sample flow front up the membrane strip (Cigic and Prosen, 2009; Monbaliu *et al*., 2007).

Table 12.1 presents an overview of immunochemical screening tests developed for the application of agricultural commodities which can be applied as feed ingredients. In general the developed methods are focused on the determination of one single toxin. Different immunochemical methods have been developed for the determination of aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), deoxynivalenol (DON), 15-acetyl deoxynivalenol (15-ADON), zearalenone (ZEN) and T2-toxin (T2). Recently new immunochemical methods were developed for the

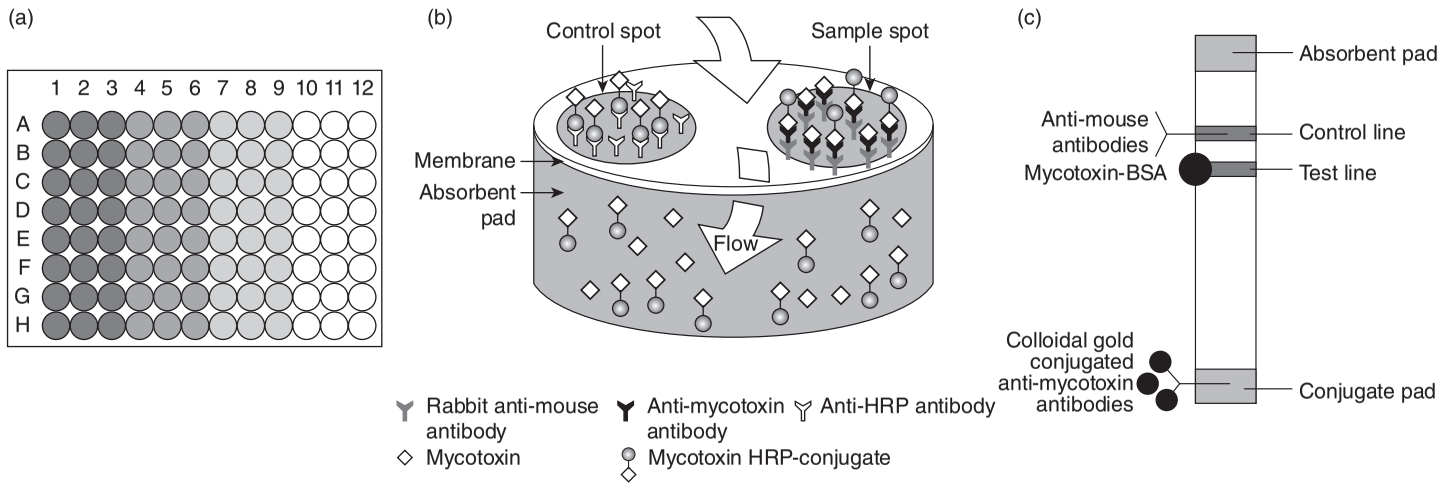

Fig. 12.2 Overview of immunochemical methods: (a) classic ELISA; (b) flow-through enzyme immunoassay (immunofiltration); (c) lateral flow immunoassay (immunostrip or dipstick). Adapted from Kolosova *et al.*, 2007, 2008.

Table 12.1 Overview of immunochemical screening methods for agricultural commodities used as feed ingredients

Toxin	Matrix	Extraction (v/v)	AB	COMP	Principle	IC_{50} ($\mu g.L^{-1}$)	LOD ($\mu g.L^{-1}$)	Recovery (%)	Reference
AF-B_1	Grain	MeOH/H_2O 70/30	M	D	ELISA Optical density	0.62	0.02	94–113	Kolosova *et al.*, 2006
AF-B_1	Corn Wheat	MeOH/H_2O 80/20	P	D	Immunofiltration		0.01	91–104	Pal and Dhar, 2004
AF-B_1	Pig feed	MeOH/H_2O 80/20	M	ID	Dipstick		5 $\mu g.kg^{-1}$		Delmulle *et al.*, 2005
AF-B_1	Corn	MeOH/PBS 85/25	P	ID	Multi-channel electrochemical detection intermittent pulse amperometry		0.03	103	Piermarini *et al.*, 2007
AF-B_1	Barley	MeOH/PBS 85/15	M	ID	Electrochemical immunosensors		0.09	100–125	Ammida *et al.*, 2006
AF-B_1	Grain	Chloroform/H_2O 125/12.5	ScFV	D	Fluorescence (FITC)		0.05		Daly *et al.*, 2002
AFs	Grain	MeOH/H_2O 70/30	M	D	Fluorescence polarization				Nasir and Jolley, 2002
AFs	Grain	MeOH/H_2O 70/30		D	ELISA		2.5 $\mu g.kg^{-1}$		Zheng *et al.*, 2005b
FB_1	Maize	PBS	M	D	Portable fluorescence polarization		500	94.3	Maragos *et al.*, 2001
FB_1	Corn Barley Oat	MeOH/H_2O 75/25	P	D	Chemi-luminescence ELISA	0.32	0.09		Quan *et al.*, 2006
FB_1	Barley		M	D	ELISA		0.5		Wang *et al.*, 2006
	Maize			D	Lateral flow colloidal gold		1		

FB_1/ FB_2	Maize	MeOH/H_2O 80/20	M	ID	Flow-through immunoassay			Paepens *et al.*, 2004
FB	Maize	MeOH/H_2O 70/30			Rapid lateral flow		199 $\mu g.kg^{-1}$	Molinelli *et al.*, 2009
OTA	Maize Barley	MeOH/H_2O 50/50	P	D	ELISA	0.90	85.9	Yu *et al.*, 2005
OTA	Wheat	ACN/H_2O 60/40	M	D	Electrochemical immunosensor	1.6 $\mu g.kg^{-1}$	0.4 $\mu g.kg^{-1}$	Alarcon *et al.*, 2006
OTA	Cereal	MeOH/PBS 70/30	M	D	ELISA		0.15	Liu *et al.*, 2007
OTA	Barley	MeOH/H_2O	P	D	ELISA	0.07	74–110	Wang *et al.*, 2007b
	Wheat Oat Corn	50/50			Flow-through colloidal gold		1	
OTA	Cereal	MeOH/H_2O 50/50	P	ID	Time resolved fluoro-immunoassay	1.018	0.02 95.8	Huang *et al.*, 2006
OTA	Grain	ACN/H_2O 5/1	P	ID	EIA		20 $\mu g.kg^{-1}$	Kononenko *et al.*, 2000
OTA	Corn Barley Wheat	MeOH/H_2O 70/30		D			1.9 $\mu g.kg^{-1}$	Zheng *et al.*, 2005a
OTA	Cereal	MeOH/H_2O 10/90	M		Surface plasmon resonance biosensor		<0.5	Yuan *et al.*, 2009

Table 12.1 *Continued*

Toxin	Matrix	Extraction (v/v)	AB	COMP	Principle	IC_{50} ($\mu g.L^{-1}$)	LOD ($\mu g.L^{-1}$)	Recovery (%)	Reference
OTA AF-B_1	Barley Wheat	ACN/H_2O 60/40	M	D	Optical waveguide lightmode spectroscopy				Adanyi *et al.*, 2007
OTA T2	Wheat Maize Barley	MeOH/H_2O 80/20	M		Membrane-based flow through		4 $\mu g.kg^{-1}$ 50 $\mu g.kg^{-1}$		De Saeger *et al.*, 2002
DON	Wheat	PBS	M	D	Portable fluorescence polarization			94.6	Maragos and Plattner, 2002
DON	Wheat	ACN	M		Surface plasmon resonance based inhibition				Tudos *et al.*, 2003
DON	Wheat	MeOH/H_2O 10/90	P	ID	Surface plasmon resonance based inhibition	720	2.5	104	Schnerr *et al.*, 2002
DON	Wheat		ScFV	D	ELISA				Wang *et al.*, 2007a
DON	Wheat	ACN/H_2O 10.5/89.5	P	ID	ELISA		2	80–125	Schneider *et al.*, 2000
DON	Wheat	PBS	M	D		18		88.7	Maragos and McCormick, 2000
ZEN	Cereal Swine feed	MeOH/ACN 50/50	P	D	Flow-through fluorescence	0.087	0.007		Urraca *et al.*, 2005
ZEN	Wheat	MeOH/H_2O 70/30	ScFV	D	ELISA		1000		Wang *et al.*, 2008
ZEN	Wheat				Open sandwich immunoassay	8.2	~1		Suzuki *et al.*, 2007
ZEN	Feed	ACN/H_2O 6/1	P	ID	ELISA		200 $\mu g.kg^{-1}$		Burkin *et al.*, 2000

ZEN	Corn	MeOH/H_2O 60/40	M		Gold nanoparticle immunochromatographic assay		30 $\mu g.kg^{-1}$		Shim *et al.*, 2009b
ZEN	Cereal	MeOH/H_2O 80/20	M	D	ELISA	1.13	0.15	87–112	Thongrussamee *et al.*, 2008
AFs OTA FB_1 ZEN	Feed	MeOH/H_2O 70/30		D	ELISA		2 $\mu g.kg^{-1}$ 1 $\mu g.kg^{-1}$ 200 $\mu g.kg^{-1}$ 10 $\mu g.kg^{-1}$		Klaric *et al.*, 2009
OTA DON	Wheat Barley Corn Maize	MeOH			Fluoroimmunoassay		65 $\mu g.kg^{-1}$ 85 $\mu g.kg^{-1}$		Ngundi *et al.*, 2006
DON ZEN	Wheat	MeOH/H_2O 80/20	M		Fluorescence polarization		1500 $\mu g.kg^{-1}$ 100 $\mu g.kg^{-1}$		Kolosova *et al.*, 2007
OTA ZEN	Corn	MeOH/PBS 30/70	M		One-step immunochromatographic striptest		2.5 5		Shim *et al.*, 2009a
ZEN T2	Feed	ACN/H_2O 80/20	M		Tandem clean-up immunofiltration column				Basova *et al.*, 2010

Abbreviations: AB: antibody; M: monoclonal; P: polyclonal; ScFV: single-chain fragment variable; COMP: competition; D: direct competition; ID: indirect competition.

simultaneous determination of different toxins, such as the simultaneous determination of OTA and ZEN in corn, of DON and ZEN in wheat, of OTA and DON in wheat, barley, corn and maize, and of ZEN and T2 in feed (Basova *et al*., 2010; Kolosova *et al*., 2007; Ngundi *et al*., 2006; Shim *et al*., 2009a). The rapid determination of mycotoxins in a complex feed matrix does not require preliminary removal of impurities. In general the mycotoxins were extracted using a mixture of methanol/water or acetonitrile/water in different proportions. Afterwards the extract was immediately applied to the test or previously diluted with a buffer solution (phosphate buffer saline (PBS) or phosphate buffer saline tween (PBST)).

Alternative techniques have been developed to overcome the drawbacks of the use of antibodies. This interesting research domain has led to different new approaches but the applications for feed are rather limited. An enzymatic spectrophotometric method for the determination of aflatoxin B (AF-B) in barley based on acetylcholinesterase (AChE) inhibition has been published. The determination is based on the capacity of AF-B to inhibit AChE, followed by a spectrophotometric measurement of the enzyme activity in the absence and presence of the toxin. The results are obtained within a few minutes (Arduini *et al*., 2007).

Confirmatory methods

An analytical confirmatory method needs a more extensive sample clean-up after the initial solvent extraction step. The extraction step is classically executed by blending or mechanical shaking of the feed matrix with a mixture of water and organic solvents combined with a modifier, like an acid or a base, to increase the extraction efficiency. As shown later in Tables 12.2 and 12.3, mixtures of methanol and acetonitrile are frequently used solvents with or without the addition of acetic acid or sodium hydrogen carbonate. The choice of the extraction solvent depends on the physicochemical properties of the mycotoxin(s) analysed, the sample matrix and the clean-up methodology used (Krska *et al*., 2007). Other approaches such as accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE) or microwave-assisted extraction (MAE), for the analysis of AFs in nuts and ZEN in wheat, corn, cereals and swine feed have been published (Carnpone *et al*., 2009; Pallaroni *et al*., 2002; Urraca *et al*., 2004). Supercritical fluid extraction (SFE) using CO_2 as an environmentally safe extraction medium has been applied for the determination of DON, 3-acetyldeoxynivalenol (3-ADON), 15-ADON, fusarenon-X (FUS-X) and nivalenol (NIV) in wheat, of DON, diacetoxyscirpenol (DAS) and T2 in feeds, of fumonisin B_1 (FB_1) in corn, of AFs in corn and of beauvericine (BEA) in maize (Ambrosino *et al*., 2004; Holcomb *et al*., 1996; Huopalahti *et al*., 1997; Josephs *et al*., 1998; Selim *et al*., 1996).

During the sample clean-up step, interfering substances are removed and the analyte is preconcentrated. Different techniques are applied, such as liquid–liquid partitioning with hexane for defatting of feed sample extracts

Table 12.2 Overview of TLC methods of the last decade for agricultural commodities used as feed ingredients

Toxin	Matrix	Extraction (v/v)	Clean-up	Plate	Mobile-phase (v/v)	Spray reagents (v/v)	Detection	LOD ($\mu g.kg^{-1}$)	Reference
AFB_1 AFB_2 AFG_1	Cereals Feed	$MeOH/H_2O$ 70/30	Chloroform filtered through anhydrous $Na_2S_2O_4$	Silica GF_{254}	Toluene/ ethylacetate/ formic acid, 5/4/1	Not applied	UV (366 nm)	2 4.3 4.3 4.3	Klaric *et al.*, 2009
AFG_2 OTA ZEN FB_1				Silica 60 $F2_{54}$	$MeOH/H_2O$, 8/2	0.5% anisaldehyde in MeOH/ acetic acid/ sulphuric acid (85/10/5)		60 250 1000	
AFB_1 AFB_2 AFG_1 AFG_2 OTA	Grain	Chloroform	Chloroform filtered through anhydrous $Na_2S_2O_4$	Silica GF_{254}	(1) Chloroform/ acetone, 9/1 (2) toluene/ ethylacetate/ formic acid, 6/3/1	20% $AlCl_3$ 20% sulphuric acid	UV (366 nm)	0.14 ng/plate 0.30 ng/plate 0.10 ng/plate 0.13 ng/plate 0.88 ng/plate	Braicu *et al.*, 2008
AFB_1 AFB_2 AFG_1 AFG_2	Maize Poultry feed	Chloroform			Toluene/ chloroform/ ethyl acetate/ formic acid, 70/50/50/20		UV		Fraga *et al.*, 2007

Table 12.2 *Continued*

Toxin	Matrix	Extraction (v/v)	Clean-up	Plate	Mobile-phase (v/v)	Spray reagents (v/v)	Detection	LOD (µg.kg^{-1})	Reference
OTA T-2 DAS	Grain Animal Feed	MeOH/H_2O 50/50	LLE: chloroform	SIL-G25	Toluene/ethyl acetate/ formic acid, 100/50/15	• 4% nicotinamide in MeOH/ acetone (83.3/16.7) • 2 acetylpyridine/ *n*-hexane (3/100) • 2N KOH/EtOH/ H_2O (11.2/96/6) • Formic acid/ ether (30/100)	Visual UV		Sokolovi and Impraga, 2006
DON	Grain Animal feed	ACN/H_2O 75/25	LLE: hexane SPE: florisil	HPTLC Kieselgel 60 plate F-254	(1) Toluene/ ethyl acetate / formic acid, 5:4:1 (2) Chloroform/ MeOH, 7:1	5% $AlCl_3$	UV		
STERIG	Cereal	ACN/4%KCl 95/5	LLE: toluene SPE: phenyl	Amino HPTLC			UV (366 nm)	2	Stroka *et al.*, 2004a
FB_1	Maize	MeOH/H_2O 75/25	SPE: SAX	LK C_{18}	MeOH/4%KCl, 70/30	Fluorescamine reagent	UV		Shephard and Sewram, 2004

Table 12.3 Overview of HPLC methods for agricultural commodities used as feed ingredients

Toxin	Matrix	Extraction (v/v)	Clean-up	Mobile phase (v/v)	Stationary phase	Detector	LOD ($\mu g.kg^{-1}$)	Reference
DON	Feed	H_2O	Sol-gel immunoaffinity	H_2O/ACN/MeOH (85/5/10)	Synergi 4u Polar-RP 250 × 4.6 mm 4 µm	UV (220 nm) *Tetramethoxy-silane*	200	Klinglmayr *et al.*, 2010
OTA	Animal feed	Chloroform	Filtration	ACN/H_2O/acetic acid (99/99/2)	Hypersil C_{18} 150 × 4.6 mm 3 µm	Fluorescence $\lambda_{ex.}$: 330 nm $\lambda_{em.}$: 450 nm	0.1 ng/mL	Aboul-Enein *et al.*, 2002
ZEN	Cereals	ACN/H_2O 90/10	ZearalaTest™ immunoaffinity	ACN/H_2O (50/50)	Cosmosil 5C_{18}-AR-II 250 × 4.6 mm 5 µm 30°C	Fluorescence $\lambda_{ex.}$: 274 nm $\lambda_{em.}$: 440 nm	2.5	Liao *et al.*, 2009
FB_1	Corn	MeOH/H_2O 3/1	SAX-SPE	MeOH/0.1M NaH_2PO_4 (77/23)	ODS 150 × 4.6 mm 5 µm	Fluorescence $\lambda_{ex.}$: 335 nm $\lambda_{em.}$: 440 nm *OPA-reagent/2-mercaptoethanol*	2	Gong *et al.*, 2009
FB_1 FB_2	Corn	ACN/H_2O 50/50	C_{18}-SPE	ACN/H_2O/acetic acid (50/50/1)	Nucleosil 100 150 × 4.6 mm 5 µm	Fluorescence *OPA-reagent 2-mercaptoethanol*	30 50	Dilkin *et al.*, 2001
FBs	Wheat	ACN/ MeOH/ H_2O 1/1/2	SAX-SPE	MeOH/0.1M NaH_2PO_4 (73/27)	Synergi MAX-RP 250 × 4.6 mm 4 µm	Fluorescence $\lambda_{ex.}$: 335 nm $\lambda_{em.}$: 440 nm *OPA-reagent*	5	Shephard *et al.*, 2005
FB_1 FB_2	Feed	ACN/ MeOH/ H_2O 25/25/50	Fumoniprep immunoaffinity	MeOH/0.1M NaH_2PO_4 (77/23)	Symmetry Sperisorb ODS 250 × 4 mm 5 µm	Fluorescence $\lambda_{ex.}$: 335 nm $\lambda_{em.}$: 440 nm *OPA-reagent/2-mercaptoethanol*	50 75	Ghali *et al.*, 2009
AF-B_1 AF-B_2 AF-G_1 AF-G_2 AF-M_1 AF-M_2 aflatoxicol	Pet food	MeOH/H_2O 60/40	EAS1-EXTRACT™ immunoaffinity	H_2O/ACN/MeOH (60/22/18)	HS-10 C_{18} 250 × 4 mm 5 µm	Fluorescence $\lambda_{ex.}$: 370 nm $\lambda_{em.}$: 418 nm *hexane/trifluoro-acetic acid*	3–7	Sharma and Marquez, 2001

Table 12.3 *Continued*

Toxin	Matrix	Extraction (v/v)	Clean-up	Mobile phase (v/v)	Stationary phase	Detector	LOD ($\mu g.kg^{-1}$)	Reference
AF-B_1 AF-B_2 AF-G_1 AF-G_2	Feed	MeOH/H_2O 80/20	Aflaclean™ Immunoaffinity	H_2O/MeOH/ACN (55/15/30)	Zorbax Eclipse XDB C_{18} 150 × 4.6 mm 5 µm	Fluorescence λ_{ex}.: 356 nm λ_{em}.: 435 nm	0.08 0.02 0.16 0.04	Muscarella *et al.*, 2009
Multi-toxin	Corn Cereals	ACN/H_2O 84/16	Florisil Sep Pak	ACN/H_2O/ 0.75% acetic acid (65/35)	Lichrospher 100 C_{18}-RP 250 × 4 mm 5 µm	Fluorescence λ_{ex}.: 292 nm λ_{em}.: 425 nm *Coumarin-3-carbonyl-chloride*	10 – 15	Jimenez *et al.*, 2000
Multi-toxin	Grain	ACN/H_2O 80/20	Mycosep225	Gradient: – H_2O – MeOH	Spherisorb S30DS2 250 × 2.1 mm 3 µm	Fluorescence λ_{ex}.: 292 nm λ_{em}.: 425 nm *Coumarin-3-carbonyl-chloride*	0.2 – 1	Dall'Asta *et al.*, 2004
CPA	Poultry feed	MeOH/1% $NaHCO_3$	Hexane (defatting) filtration	Gradient: – MeOH/5% ammonium acetate (30/70) – MeOH	Symmetry C_{18} 150 × 2.1 mm 5 µm	MS/MS ESI^-	5	Moldes-Anaya *et al.*, 2009
OTA	Cereal	ACN/H_2O 60/40	Ochrastar immunoaffinity	0.1% FA/MeOH/ACN (1/1/1)	Xterra RP18 250 × 3 mm 5 µm	MS/MS ESI^+	0.012	Chung and Kwong, 2007
STERIG	Wheat	ACN/H_2O 84/16	Strata X – SPE	ACN/H_2O (75/25), 0.01% FA	Luna C_{18} 150 × 4.6 mm 5 µm	MS/MS	0.5	Versilovskis *et al.*, 2007
Multi-toxin	Grain	ACN/H_2O 84/16	Mycosep 227 Mycosoep 216	Gradient – 1 mM ammonium acetate/ACN (80/20) – ACN	Hypersil C_{18} 200 × 2.1 mm 5 µm	MS/MS $APCI^+$	50* – 85*	Razzazi-Fazeli *et al.*, 2002
Multi-toxin	Feed	ACN/H_2O + 0.1% FA 9/1	Hexane (defatting) filtration	Gradient – H_2O + 0.05 mM ammonium acetate – MeOH + 0.05 mM ammonium acetate	Symmetry C_{18} 150 × 2.1 mm 5 µm	MS/MS APCI	5 – 20	Rundberget and Wilkins, 2002
Multi-toxin	Maize	ACN/H_2O 75/25	Carbograph-4	Gradient – H_2O – ACN/MeOH (70/30)	Alltima LC-18 150 × 4.6 mm 5 µm	MS/MS ESI^-	1.5 – 10	Lagana *et al.*, 2003

Multi-toxin	Maize	ACN/H_2O 84/16	Mycosep 227	Gradient – H_2O/ACN/MeOH (82/9/9) – H_2O/ACN/MeOH (40/60/0)	Polar-RPC$_{18}$ 150 × 4.6 mm 4 µm	MS/MS APCI$^-$	50* – 150*	Razzazi-Fazeli *et al.*, 2003
Multi-toxin	Maize	ACN/H_2O 75/25	SAX-SPE Mycosep 226	Gradient – H_2O/ACN (99/1) 5 mM ammonium acetate pH 4 – ACN	Shiseido Capcell Pak C_{18} AQ HPLC column 150 × 4.6 mm 5 µm 30°C	MS/MS APCI	3 – 20	Royer *et al.*, 2004
Multi-toxin	Wheat	ACN/H_2O 84/16	Mycosep 230		Aquasil RP-C$_{18}$ 100 × 4.6 mm 3 µm	MS/MS APCI$^-$		Berthiller *et al.*, 2005a
Multi-toxin	Maize	ACN/H_2O 84/16	Mycosep 226	Gradient – MeOH/H_2O (20/80) – MeOH/H_2O (90/10) + 5 mM ammonium acetate	Aquasil RP-C$_{18}$ 100 × 4.6 mm 3 µm	MS/MS APCI Polarity switching	0.3 – 3.8	Berthiller *et al.*, 2005b
Multi-toxin	Feed	ACN/H_2O 85/15	Mycosep 226	Gradient – H_2O – MeOH	SP-RP18-Zorbax 150 × 3 mm 3.5 µm	MS/MS	10*	Biselli and Hummert, 2005
Multi-toxin	Cereals	ACN/H_2O 84/16	Mycosep 227	Gradient – H_2O 0.00184 mM NH_3 0.13 ammonium acetate – ACN	Synergi Fusion RP 250 × 2 mm 4 µm 25°C	MS/MS ESI Polarity switching	0.2 – 4.4	Klotzel *et al.*, 2005
Multi-toxin	Grain	ACN/H_2O 84/16	C_8-SPE	ACN/MeOH/0.01 M ammonium acetate (45/45/10)	Luna C$_{18}$ 150 × 3 mm 5 µm	MS/MS ESI$^+$	0.2 – 1.5	Jestoi *et al.*, 2005
Multi-toxin	Cereals	ACN/H_2O/acetic acid 79/20/1	Dilution	Gradient – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (10/89/1) – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (97/2/1)	Gemini C$_{18}$ 150 × 4.6 mm 5 µm	MS/MS ESI	0.03 – 170	Sulyok *et al.*, 2006 (Sulyok *et al.*, 2007)

Table 12.3 *Continued*

Toxin	Matrix	Extraction (v/v)	Clean-up	Mobile phase (v/v)	Stationary phase	Detector	LOD ($\mu g.kg^{-1}$)	Reference
Multi-toxin	Maize	PBS	Multi-analyte IAC AOZFDT2™ column	Gradient – H_2O, 0.5% acetic acid, 1 mM ammonium acetate – MeOH, 0.5% acetic acid, 1 mM ammonium acetate	Gemini C_{18} 150 × 2 mm 5 µm 40°C	MS/MS	0.3 – 4.2	Lattanzio *et al.*, 2007
Multi-toxin	Cereals	ACN/H_2O 84/16	Mycosep 226	Gradient – H_2O ESI^+: 10 mM ammonium acetate ESI^-: 0.1% NH_3 – MeOH	Acquity BEH C_{18} 100 × 2.1 mm 1.7 µm	MS/MS ESI	0.003 – 0.212	Ren *et al.*, 2007
Multi-toxin	Horse feed	ACN/H_2O 84/16	Dilution	Gradient – H_2O, 1 mM ammonium formate, 20 µL/L FA – H_2O/MeOH (5/95), 1 mM ammonium formate, 20 µL/L FA	Acquity UPLC BEH C_{18} 100 × 2.1 mm, 1.7 µm 40°C	MS/MS		Mol *et al.*, 2008
Multi-toxin	Wheat Maize Silage	ACN/H_2O 80/20	Dilution Filtration	Gradient – H_2O + 0.1% FA – ACN + 0.1% FA	Alltima C_{18} 150 × 3.2 mm 5 µm	MS/MS ESI	0.5 – 200	Spanjer *et al.*, 2008
Multi-toxin	Wheat Maize	ACN/H_2O/acetic acid 79/20/1	Dilution	Gradient – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (10/89/1) – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (97/2/1)	Luna C_{18} 150 × 2 mm 5 µm 25°C	MS/MS ESI^+ (triple quadrupole)	0.5 – 800	Herebian *et al.*, 2009
Multi-toxin	Wheat Maize	ACN/H_2O/acetic acid 79/20/1	Dilution	Gradient – H_2O, 0.1% FA, 5 mM ammonium acetate – ACN, 0.1% FA	C_{18} gravity capillary column 150 × 0.3 mm 3 µm	MS/MS ESI^+ (Orbitrap)	0.4 – >2000	

Multi-toxin	Grain Wheat Barley Oat	ACN/H_2O 90/10	Filtration	Gradient SYSTEM I – H_2O, 0.2% FA – ACN, 0.2% FA SYSTEM II – H_2O, 1 mM ammonium acetate – ACN	Inertsil ODS-EP 150 × 2.1 mm 5 µm	MS/MS	0.5 – 300	Kokkonen and Jestoi, 2009
Multi-toxin	Maize silage	MeOH/H_2O 80/20	Oasis HLB-SPE	Gradient – ACN – H_2O/0.5% acetic acid	Zorbax SB-C_{18} 150 × 2.1 mm 5 µm	MS/MS	1.5 – 6.5	Richard *et al*., 2009
Multi-toxin	Maize silage	QuEChERS	Filtration	Gradient ESI^+ – H_2O, 0.4 mM ammonium acetate, 0.2% FA – ACN ESI^- – H_2O, 0.2% FA – ACN	Gemini C_6-phenyl 100 × 2 mm 3 µm	MS/MS ESI^+ ESI^-	1 – 739	Rasmussen *et al*., 2010
Multi-toxin	Feed	ACN/H_2O/acetic acid 79/20/1	C_{18} -SPE Multisep 226 filtration	Gradient – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (5/94/1) – MeOH/H_2O/acetic acid + 5 mM ammonium acetate (97/2/1)	Symmetry C_{18} 150 × 2.1 mm 5 µm	MS/MS ESI^+		Monbaliu *et al*., 2010
Multi-toxin	Cereals	QuEChERS	Dilution	Gradient SYSTEM I - H_2O 5 mM ammonium acetate pH 5.6 – MeOH SYSTEM II – H_2O 5 mM ammonium formate, 0.1% FA, pH 2.7 – MeOH	Acquity UPLC HSS T3 100 × 2.1 mm 1.8 µM 40°C	TOF-MS/MS	5 – 50	Zachariasova *et al*., 2010

* Limit of quantification, LOQ.

(Jimenez *et al*., 2000; Moldes-Anaya *et al*., 2009; Monbaliu *et al*., 2010; Rundberget and Wilkens, 2002; Sewram *et al*., 1999; Solfrizzo *et al*., 1998; Tanaka *et al*., 2000) and SPE, where the sample extract is applied to a disposable cartridge packed with different kinds of materials such as silica gel, octadecylsilane (C_{18}), aminopropyl (NH_2), florisil, ion exchange materials or mixtures of these sorbents. At present, classical SPE columns (C_{18}, strong anion exchange (SAX), NH_2) as well as the newer Mycosep™ and Multisep™ columns, i.e. columns made of several absorbents to remove interfering matrix components resulting in a clear extract without additional rinsing steps, are frequently used for feed analysis (Turner *et al*., 2009).

Another approach is the use of very selective antibody-based immunoaffinity columns. This is a very attractive clean-up method for determining a single analyte or a limited number of analytes in a complex feed matrix which contains potential interferences. At present there are immunoaffinity columns commercially available for AFs, OTA, FBs, ZEN, DON, T2, HT-2 toxin (HT2) and citrinin (CIT) (Senyuva and Gilbert, 2010). During the last decade, efforts have been made to develop and apply multi-immunoaffinity columns containing mixed antibodies on the same column. Such a column has been applied for the determination of AFs, OTA and ZEN in grains and for the determination of AFs, OTA, FB_1, fumonisin B_2 (FB_2), DON, ZEN, T2 and HT2 in maize kernels (Gobel and Lusky, 2004; Lattanzio *et al*., 2007). But the restrictions of the antibody technology have led to the development of novel techniques such as the emerging, cheap and very promising molecularly imprinted polymers (MIPs). The MIP technology (Fig. 12.3) is based on the 'key to lock' complement caused by the presence of a highly specific structural framework that provides a conformational complement to the imprint or mycotoxin (De Smet *et al*., 2009). This has led to the development of a MIP for the clean-up of cereals and swine feed for the determination of ZEN (Urraca *et al*., 2006).

Recently developed alternative approaches for sample clean-up strategies are matrix–solid phase dispersion (MSPD), solid phase micro extraction (SPME), and the quick, easy, cheap, effective, rugged and safe (QuEChERS) method. These methods combine the extraction and sample clean-up in one single step. A method for the determination of AFs in peanuts and OTA in cereals using MSPD as clean-up and the analysis of *Fusarium* toxins in cereals using the QuEChERS method have been published (Blesa *et al*., 2003, 2004; Zachariasova *et al*., 2010). Recently a QuEChERS multi-mycotoxin LC-MS/MS method has been published for the analysis of mycotoxins produced by maize and maize silage contaminants (Rasmussen *et al*., 2010). Until now no SPME methods have been published for application in feed commodities.

After the clean-up procedure, samples are analysed using different techniques such as TLC, GC and HPLC coupled to a UV detector, a fluorescence detector or a mass spectrometer. The use of TLC analysis for mycotoxins is still popular for both quantitative and semi-quantitative

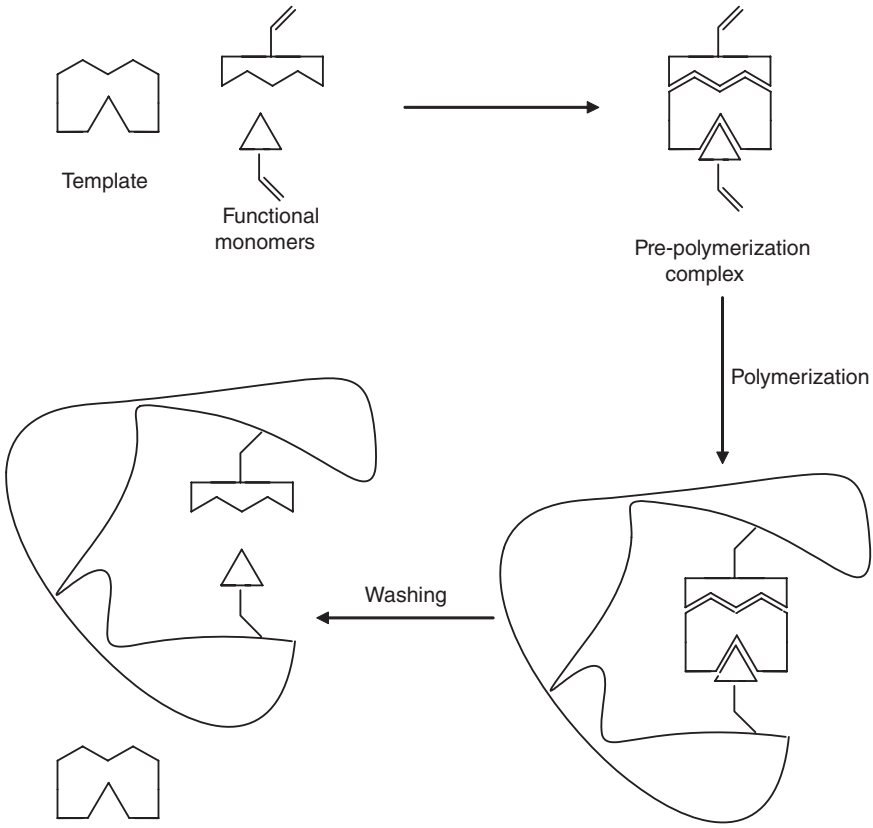

Fig. 12.3 Schematic overview of the MIP principle.

purposes due to its high throughput of samples, low operating cost and ease of identification of target compounds. A summary of TLC methods for feed commodities applied during the last decade is presented in Table 12.2. The use of two-dimensional TLC analysis is often required to remove the interfering substances of the complex feed matrix (Krska *et al*., 2007). The focus on mycotoxin determination during the last decade is changing to multi-mycotoxin research. Because recent investigations were focused on the simultaneous occurrence of different types of toxins, TLC methods for multiple detection were developed (Braicu *et al*., 2008; Fraga *et al*., 2007; Klaric *et al*., 2009; Sokolovi and Improga, 2006). In the last years TLC has been replaced more and more by GC or HPLC since these techniques offer advantages such as automation, high-performance separation and lower detection limits.

Despite modern gas chromatography combining superior separation on a capillary column with a variety of general and specific detectors, the methods published for the determination of mycotoxins in feed commodities are less numerous and are mainly focused on the determination of trichothecenes. The main disadvantages of GC analysis are the required thermal stability, the low polarity and the volatility of the target compounds. Because mycotoxins are non-volatile, derivatization by silylation or polyfluoroacylation during the analysis is often necessary for detection (Cigic and Prosen, 2009; Croteau *et al*., 1994; Kotal *et al*., 1999; Krska *et al*., 2007; Lauren and Agnew, 1991; Mirocha *et al*., 1998; Moller and Gustavsson, 1992; Mossoba *et al*., 1996; Onji *et al*., 1998; Schollenberger *et al*., 1998; Schwadorf and Muller, 1991; Seidel *et al*., 1993; Tacke and Casper, 1996; Tanaka *et al*., 2000; Turner *et al*., 2009; Weingaertner *et al*., 1997). In 2008 a method was published for the determination of trichothecenes in wheat grain without sample clean-up using comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (Jelen and Wasowicz, 2008).

At present the method of choice for mycotoxin determination is HPLC coupled to different kinds of detectors. Table 12.3 presents a summary of published HPLC methods of the last decade. To introduce or enhance fluorescence properties for fluorescence detection, tetramethoxysilane, *o*-phthaldialdehyde with 2-mercaptoethanol and cyclodextrins are frequently used agents. Only AFs, OTA, ZEN and CIT have natural fluorescence properties (Cigic and Prosen, 2009; Maragos *et al*., 2008). To overcome the drawbacks of derivatization, LC-MS has been applied as a highly reliable analyte confirmation tool and therefore has become a routine technique in feed analysis. This technical and instrumental progress has also had an increasing impact on the expanding field of mycotoxin analysis, particularly in the development of multi-mycotoxin methods. Internal standards are frequently used to compensate for ion suppression or enhancement caused by matrix compounds during LC-MS/MS analysis. However, deuterated (Herebian *et al*., 2009; Razzazi-Fazeli *et al*., 2002; Royer *et al*., 2004; Rundberget and Wilkins, 2002) and isotopically labelled (Zachariasova *et al*., 2010) internal standards are the first choice for application, but their high costs have led to the use of cheaper metabolites such as zearalanone (ZAN) (Berthiller *et al*., 2005b; Biselli and Hummert, 2005; Monbaliu *et al*., 2010; Ren *et al*., 2007; Zollner *et al*., 1999) and deepoxydeoxynivalenol (DOM) (Klotzel *et al*., 2005; Monbaliu *et al*., 2010). The first developed multi-methods focused on the determination of several mycotoxins being part of one class of mycotoxins (*Aspergillus*, *Penicillium* or *Fusarium* toxins) followed by methods for the simultaneous determination of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* toxins. Further evolution led to newly published multiple methods, including also mycotoxin metabolites, masked mycotoxins and ergot alkaloids (Monbaliu *et al*., 2010).

12.2.5 Method validation

Analytical methods have to be subjected to validation procedures to demonstrate that the method produces reliable results. In view of legal actions, trade specifications as well as monitoring or risk assessment studies, these methods need to provide accurate, repeatable and reproducible results within and between different laboratories. Any method proposed to become official for legal cases or for international trade purposes must be validated in a collaborative trial study, resulting in defined method performance characteristics. It must be emphasized that method performance requirements for the use in official control only make sense when legislative limits exist for the specific mycotoxin (Anklam *et al.*, 2002). Currently, maximum limits for AFs, OTA, patulin (PAT), DON, ZEN and FBs in foodstuffs are established in Commission Regulation 2006/1881/EC. T2 and HT2 are mentioned in this legislation but no maximum levels are established yet. Commission Regulation 2007/1126/EC amends the maximum levels for *Fusarium* toxins (DON, ZEN and FBs) in maize and maize products. Performance criteria are established for analytical methods for the official control of the maximum level of mycotoxins in foodstuffs in Commission Regulation 2006/401/EC. As mentioned previously (Section 12.2.2) only maximum levels for AF-B_1 in animal feed and maximum recommended levels for DON, ZEN, OTA and FBs are established for products intended for animal feeding (Commission Directive 2003/100/EC; Commission Recommendation 2006/576/EC). T2 and HT2 are mentioned in this recommendation but no maximum recommended levels are established. Contrary to analytical methods for food analysis, there is no specific legislation available with performance criteria for analytical methods for feed analysis. Because mycotoxins are listed as environmental contaminants in group B of Council Directive 1996/23/EC, this legislation should be applied for the official control of mycotoxins in animal feed. Therefore the performance criteria of analytical methods for feed analysis have to fulfil the performance criteria established in Commission Decision 2002/657/EC. Depending on the method's purpose, different validation parameters have to be characterized. For a qualitative screening method, the detection capability (CCβ) and the specificity are the most important validation parameters, whereas for a quantitative confirmation method CCβ, the decision limit (CCα), the trueness, the precision and the specificity have to be determined.

The half-maximum inhibitory concentration (IC_{50}), the detection limit (LOD), the cut-off and the recovery are the most validated parameters for screening methods. The specificity of screening methods is tested by investigating the cross-reactivity (CR). This is calculated by comparing the inhibition concentrations of the mycotoxin of interest and the competitive analyte using the following formula:

$$\mathrm{CR}(\%) = \left[\frac{\mathrm{IC}_{50}\ \text{mycotoxin}}{\mathrm{IC}_{50}\ \text{competitive analyte}} \right] \times 100$$

The LOD, the limit of quantification (LOQ), the precision (the intra-day as well as the inter-day precision), the trueness, the linearity, the recovery and matrix effects are the most frequently determined parameters of a quantitative confirmatory method. Because of the lack of uniform international validation guidelines, different approaches are used to calculate certain validation parameters. For example, the limit of detection can be established visually (with the presentation of relevant chromatograms) or using the signal-to-noise ratio, or it can be calculated using a calibration curve, resulting in a different sensitivity of the method (Araujo, 2009).

Recently more and more attention is being paid to the measurement uncertainty. It is recommended to report the result of the analysis in combination with the expanded measurement uncertainty to describe quantitatively the imperfection of the results of the laboratory, which increases the reliability of the obtained result (ISO/IEC 17025, 2005).

Most validation studies are performed using samples spiked with a known mycotoxin concentration. It is more correct to use certified reference materials (CRMs), but these are not available for all matrices and mycotoxins of interest.

12.2.6 Future trends

The worldwide occurrence of mycotoxins will lead to the development of techniques and methods which are based on the newest technologies. New screening methods and confirmatory methods will be developed to analyse more mycotoxins and more samples and will focus on easier, cheaper and environmentally friendly procedures. All these efforts will be made to obtain better insights into the real mycotoxin problem and to prevent human and animal exposure to mycotoxins.

At present it is possible with recombinant technology to isolate the variable portions of the immunoglobulin that are primarily involved with antigen binding, such as the single-chain variable fragments (ScFV) where the variable domains of the light chain and the heavy chain have been joined with a synthetic peptide linker (Fig. 12.4). The ScFV are produced in large quantities in bacterial expression systems at low cost, which makes the recombinant technology very promising (Maragos, 2009). Table 12.1 presents the application of ScFV for the determination of aflatoxin B_1 (AF-B_1) in grain, and for DON and ZEN in wheat (Daly *et al*., 2002; Wang *et al*., 2007a, 2008). The development of enzymatically mediated electrochemical sensors, DNA sensors and MIP technology will lead to new applications in the field of mycotoxin feed analysis (Prieto-Simon *et al*., 2007). The unavoidable co-occurrence of different types of mycotoxins in feed is going to lead to the development of multi-mycotoxin rapid screening tests (Goryacheva *et al*., 2007). Recently OTA in wheat was determined with a DNA aptamer. Aptamers are synthetic, single-stranded oligonucleotides that bind with high affinity and specificity to molecular

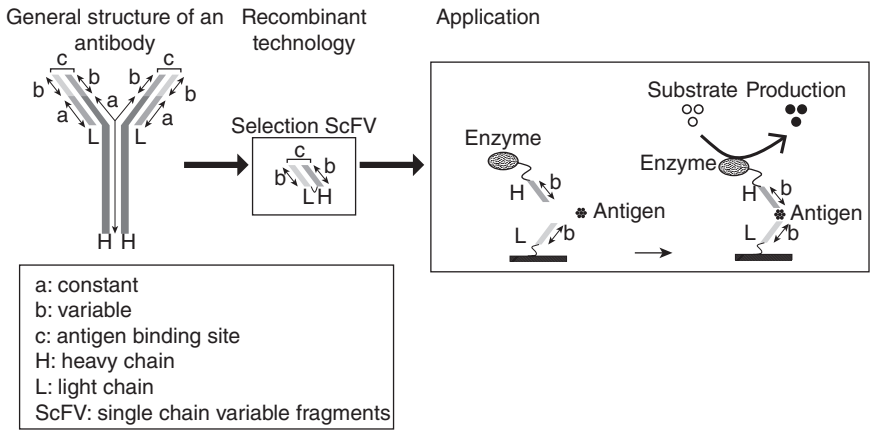

Fig. 12.4 Principle of the application of single-chain variable fragments in an open sandwich immunoassay. Adapted from Suzuki *et al*., 2007.

targets (Cruz-Aguado and Perner, 2008). The introduction of this innovative technology will lead to the development of new applications in mycotoxin research.

At present confirmatory methods are focused on faster analysis, using fewer extraction solvents and fewer time-consuming clean-up strategies. There is also a growing interest in masked mycotoxins, which are mycotoxin derivatives via conjugation with more polar substances such as sugars, amino acids or sulphate groups. Zearalenone-4-glucoside, deoxynivalenol-3-glucoside and bound FBs are the main known representatives. The masked mycotoxins can easily escape established routine analyses because of their different chemical behaviour. However, after hydrolysis in the digestive tracts of humans and animals by enzymatic processes or an alkaline environment, toxic effects can be caused (Galaverna *et al*., 2009). The development and application of methods for the analysis of masked mycotoxins will lead to better insights into the real mycotoxin problem in animal feed.

The introduction and application of time-of-flight mass spectrometers offer new opportunities in the field of mycotoxin research. Despite the tandem mass spectrometry technology, where the retention characteristics of selected mycotoxins and their specific fragmentation must be known or previously determined using available standards, it offers advantages in selectivity, though it is of limited use for the identification of non-target mycotoxins and other fungal metabolites. The untargeted screening can be accomplished by using spectral databases and/or molecular weight and fragment information from accurate mass determinations (Pico *et al*., 2007; Senyuva *et al*., 2008).

12.3 Detection and determination of plant toxins

Many plants produce chemicals with a wide range of mammalian toxicity and a risk to animal and human consumers of food that may be contaminated by such plant toxins. New plant-associated intoxications are continuing to be reported and causative toxins are chemically identified (Than *et al*., 2005). Problems associated with the detection and quantification of plant toxins in animal feed are similar to those encountered with the analysis of other analytes, including sampling, method validation and technological requirements. Screening assays with high throughput capacity but possible false negative and false positive results should be supported by confirmatory chromatographic assays, which are more expensive and time-consuming. This section will focus on four classes of plant toxins, i.e., pyrrolizidine alkaloids, saponins, glucosinolates and cyanogenic compounds.

12.3.1 Pyrrolizidine alkaloids

The hepatotoxic pyrrolizidine alkaloids (PAs) are mono-, di- or macrocyclic diesters of necine or otonecine bases and also occur as their respective *N*-oxides. More than 350 PAs are described and are produced as secondary metabolites of more than 6000 plant species occurring worldwide, including the families of Boraginaceae, Compositae (Asteraceae) and Leguminosae (Fabaceae). The pattern of PAs in plants varies greatly, depending on the plant variety, climatic conditions, period of sampling and parts of the plant analysed. Livestock can be exposed to pyrrolizidine alkaloids in their natural pasture or through feed with pyrrolizidine alkaloid-containing plants. Besides being liver toxins, the PAs are potential carcinogens and genotoxins (Than *et al*., 2005) (for more details see Chapter 14).

The European Food Safety Authority (EFSA) opinion on PAs (European Food Safety Authority, 2007b) highlights the importance of quantitative chemical analysis of the specific PAs presumed responsible for the toxic effects. An overview of existing methods of analysis is presented in the EFSA opinion and summarized here. Recently, a further update and comprehensive review on analytical methods for toxic PAs was published by Crews *et al*. (2010). Plant materials are extracted in cold or hot methanol (with or without acidification), and after evaporation PAs are dissolved in diluted aqueous acids to which zinc is added to reduce the *N*-oxides. The zinc is then removed by filtration and the pH is adjusted before the alkaloids are extracted using organic solvents or, in case of polar PAs, with saturated potassium carbonate solutions. Further sample clean-up includes liquid–liquid partitioning or SPE techniques. SPE based on cation-exchange resins is the most popular and has the advantage that PAs and their *N*-oxides can be simultaneously isolated without interfering co-extractants. GC-MS with fused silica columns has been considered for a long time as the most sensitive detection method for PAs. However, this method is not suitable

for the detection of *N*-oxides which are unstable under GC conditions, and multiple analyses of the same sample, derivatized and underivatized, are necessary. HPLC coupled to MS is nowadays the most frequently applied method. HPLC-MS is suitable for the simultaneous analysis of PAs in combination with their *N*-oxides, without prior reduction or derivatization and with limits of detection comparable to GC-MS (Than *et al*., 2005). A major drawback for the quantitative analysis of PAs is the lack of commercially available standards (Crews *et al*., 2010). As screening methods, ELISA methods based on polyclonal antibodies against PAs have been developed. The Australian CSIRO Plant Toxin Research group developed two separate antisera against the groups of PAs found in species of the plant genera *Echium* and *Heliotropium*, and one antisera against the group of PAs found in some species of both the *Senecio* and *Crotalaria* genera. However, the developed antisera do not effectively cross-react with the *N*-oxide and an additional reduction step is necessary to convert *N*-oxides to free bases prior to ELISA, which may impair the accuracy in the detection of other PAs (Than *et al*., 2005).

12.3.2 Saponins

Saponins are a diverse group of widely distributed low-molecular-weight secondary plant metabolites, present in more than 100 plant families. The name 'saponins' derives from the Latin word for soap because of the stable foam which is formed in aqueous solutions. Saponins can be classified into two groups: pentacyclic triterpenoid saponins and steroidal saponins. Steroidal saponins are mainly found in monocotyledons (Agavaceae, Dioscoreaceae and Liliaceae), while triterpenoid saponins mostly are present in dicotyledons (Fabaceae, Araliaceae and Caryophyllaceae). Saponins can have anti-nutritional effects and may cause toxic effects if present in food or feed, while on the other hand they may also have beneficial health effects. Significant concentrations of saponins are found in some food and feed plants such as *Madhuca* species, alfalfa (*Medicago sativa* L.), soybean (*Glycine max*), quinoa (*Chenopodium quinoa* Willd.), and sugar beet (*Beta vulgaris* L.). The saponin content of plant materials is affected by the plant species, the genetic origin, the part of the plant being examined, the environmental and agronomic factors, and post-harvest treatments (storage and processing) (Güçlü-Üstündag and Mazza, 2007).

The EFSA opinion on saponins in *Madhuca longifolia* (European Food Safety Authority, 2009) highlights the importance of quantitative chemical analysis of the specific compound(s) presumed responsible for the toxic effects. An overview of existing methods of analysis is presented in the EFSA opinion and summarized here. Due to the ability of certain saponins to facilitate the formation of foam/emulsions, care must be taken during the extraction and clean-up steps. Saponins are surface active compounds because of the presence of a lipid-soluble aglycone and water-soluble sugar

chain(s) in their structure, and form micelles in aqueous solutions above a critical micelle concentration. They are characterized by an inherent relatively low solubility, both in water and in a number of more lipophilic solvents, and are therefore difficult to keep in solution for analysis. A comprehensive review on the physicochemical properties of saponins was published by Güçlü-Üstündag and Mazza (2007). Saponins are traditionally extracted into water/ethanol mixtures, after which the ethanol is removed by evaporation and the saponins extracted from the water phase into 1-butanol. Considerable efforts have been made to improve this methodology (e.g. the use of supercritical CO_2 extraction has proven successful) and these extraction studies were reviewed by Güçlü-Üstündag and Mazza (2007). Both normal phase and reversed phase HPLC methods are used for the separation of saponins. As most of the saponins do not possess chromophoric groups, either spectrophotometric detection with pre-column derivatization (with e.g. benzoyl chloride) or detection with mass spectrometry has been used. For screening purposes monoclonal antibody based ELISA methods have been described for some saponins. A comprehensive review on chromatographic determination of plant saponins was published by Oleszek and Bialy (2006). Still, there is an urgent need to improve existing methods, to develop simple and specific methods for the quantification of saponins in feed and to make reference standards available.

12.3.3 Glucosinolates

Glucosinolates (GLS) (alkyl aldoxime-*o*-sulphate esters with a β-D-thioglucopyranoside group) are secondary plant metabolites of species belonging to the family of Brassicaceae. Many common vegetables such as broccoli, Brussels sprouts, cabbage and cauliflower belong to this plant family. At the same time the genera *Brassica*, *Crambe*, *Sinapis* and *Raphanus* include important oil- and protein-rich agricultural crops used for the production of plant oils, such as rapeseed oils. The press cake is used as an animal feed ingredient. According to differences in their structure, GLS have been classified as aliphatic, aromatic, -methylthioalkyl and heterocyclic (indole-) GLS. Glucosinolate producing plants as well as some microorganisms contain specific β-thioglucosidases (myrosinases). Upon plant damage (including chewing during ingestion) the myrosinases are released and convert the GLS into diverse breakdown products, such as isothiocyanates, oxazolidinethiones, thiocyanates, nitriles, epithionitriles and other indol-3-ylmethyl derivatives. The biological effects of plant GLS in mammalian species are predominantly related to these glucosinolate-derived compounds. Biological effects can be toxic (changes in thyroid function), anti-nutritional or beneficial to health (anti-carcinogenic), depending on the structures and concentrations of the GLS.

The EFSA opinion on GLS (European Food Safety Authority, 2008) highlights the importance of quantitative chemical analysis of the specific

substances responsible for the toxic effects. An overview of existing methods of analysis is presented in the EFSA opinion and summarized here. The most rapid method to estimate the concentration of total GLS is an enzymatic assay in which GLS are hydrolysed by commercially available myrosinase and indirectly measured (spectrophotometrically) from the glucose content. Although GC methods have also been used for analysis of GLS, HPLC is at present the most widely used method. The analysis of de-sulphoglucosinolates (enzymatically desulphated) by HPLC is recommended by the International Organization for Standardization (ISO), as described in ISO standard 9167-1 for rapeseed-specific GLS. Rapeseed reference material containing the de-sulphoglucosinolates is available at the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The methods comprising a desulphation step, however, do not allow the analysis of those GLS which have substituents on the thioglucose part, or which have acidic groups in the side chain. Therefore, several other analytical methods focus on the determination of intact GLS either by HPLC or by capillary electrophoresis. For confirmatory analyses, HPLC separation can be combined with MS. Hydrophilic interaction liquid chromatography (HILIC) can be used for the separation of polar GLS, while ion-pair chromatography is used for non-polar GLS (Smiechowska *et al*., 2010). Clarke (2010) reviewed GLS analysis in food, with focus on aspects of extraction, ultraviolet (UV) and MS parameters, UV response factors, quantification procedure, availability of stable isotope labelled internal standards, and certified reference materials. An electronic database of structures, formulae and accurate masses of 200 known and 180 predicted GLS is provided for use in MS. Smiechowska *et al*. (2010) recently published an overview introducing advantages and disadvantages of different methods of determination of glucosinolates, isothiocyanates and indoles present in cruciferous vegetables. Njumbe Ediage *et al*. (2010) proposes an analytical strategy based on a combination of LC–electrospray ionization (ESI)–MS/MS, LC–atmospheric pressure chemical ionization (APCI)–MS/MS, and HPLC–photo diode array (PDA) for screening, identification and quantification of GLs in black radish based dietary supplements. Because of the complex nature of feed and the limited availability of reference materials, to obtain reliable and credible information on the presence and amount of GLS is a serious challenge and certainly requires combined techniques.

12.3.4 Cyanogenic compounds

Cyanogenic glycosides are produced as secondary metabolites by various plant species and include compounds such as amygdalin, dhurrin, linamarin, linustatin, lotaustralin, neolinustatin, prunasin and sambunigrin (Rietjens *et al*., 2005). Hydrogen cyanide (HCN) is formed from these cyanogenic glycosides by hydrolytic enzymes. Crushing of plant materials either by technical processes or by chewing initiates this enzymatic hydrolysis. Hydrolysis to

release HCN can also be accomplished by microorganisms in the gastrointestinal tract. Cyanogenic glycosides are found in several common feed ingredients such as sorghum, cassava, linseed and a number of forage legume species including some clovers and grasses. Exposure to HCN as well as to the cyanide anion (CN^-) may lead to acute, fatal intoxications in all animal species. More frequently, however, chronic intoxications are observed, characterized by growth depression and neurological symptoms.

The EFSA opinion on cyanogenic compounds (European Food Safety Authority, 2007a) identified the need for up-to-date analytical methods that allow the determination of the total cyanogenic potential (TCP) by ensuring full conversion to the toxic form before analysis, in order to reflect the maximum possible concentration of HCN. One method for the determination of cyanide (free and combined in the form of glycosides) is officially approved for use in feedingstuffs (products derived from flaxseed, manioc flour and certain species of beans) within the EU (Directive 71/250/EEC). Compounds to be detected and quantified in feed include the cyanogenic glycosides and the cyanohydrins (α-hydroxynitriles), together with the total pool of cyanide (HCN/CN^-). The TCP or cyanogenic potential (CNp) is determined based on hydrolysis of the glycosides, degradation of the cyanohydrins to form cyanide and subsequent measurement of total cyanide. A wide range of relatively simple assays have been developed for this purpose and further optimized, but the major challenge still remains the reliable and complete hydrolysis of the glycosides. When including one or more chromatographic separations, one could also quantify each of the compounds present in the sample, i.e. each glycoside and cyanohydrin together with the concentration of cyanide. Such an analysis gives a more detailed picture of the toxins present. Finally, the TCP or CNp should be reported and expressed in terms of HCN equivalents.

Sample handling (storage, comminution, extraction) is a crucial step and precautions must be taken to avoid loss of cyanide and cyanohydrins, which are quite unstable. Cyanide is best preserved (as CN^-) in aqueous media, while cyanohydrins are quite stable at acidic pH or in organic solvents. Certain enzymes, such as glycosidases and cyanohydrin lyases may be present in the plant material and these enzymes must be kept inactive during comminution and extraction. This can be done by using low handling temperatures or by irreversible inactivation. Hydrolysis of cyanogenic glycosides in the prepared feed extracts is done either by acid catalysis or by enzymatic degradation. Total hydrolysis must be ensured, but the use of autolysis in a fresh plant material homogenate is not recommended for this purpose. When extraction and hydrolysis are complete, the cyanide formed can be quantified by different methods including titration, spectrophotometry (colorimetry, fluorometry and chemiluminescence), atomic absorption spectrometry, ion chromatography, and HPLC and GC with different detectors. Bacala and Barthet (2007) reported the development and validation of a rapid methodology for the extraction and GC analysis of the cyano-

genic glycosides linustatin and neolinustatin from flaxseed with phenyl-beta-D-glucopyranoside as an internal standard.

12.4 References

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13

Prevention and control of animal feed contamination by mycotoxins and reduction of their adverse effects in livestock

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Abstract: The economic impact of mycotoxins on animal agriculture is substantial. Prevention of mycotoxicoses in livestock and poultry through specialized non-nutritive feed additives has gained wide acceptance. This chapter discusses various dietary strategies utilized to counteract the adverse effects of mycotoxins such as inorganic and organic adsorbents, mycotoxin inactivators and nutritional supplements. The mode of action of mycotoxin detoxifying and decontaminating agents and their relative efficacies are also discussed.

Key words: mycotoxins, inorganic adsorbents, organic adsorbents, nutritional supplements.

13.1 Introduction

The worldwide economically important mycotoxins in feeds and foods are aflatoxins (AF), ochratoxins (OT) and *Fusarium* mycotoxins including trichothecenes, zearalenone (ZEN) and fumonisins (Varga and Tóth, 2005). Economic losses may occur due to (a) crop yield or production losses due to diseases caused by infestation, (b) condemnation of the agricultural products, (c) losses due to reduced animal productivity arising from mycotoxicoses, and (d) veterinary care costs (Hussein and Brasel, 2001). Other costs associated with mycotoxin contamination of feed materials include increased labour for prevention, sampling, mitigation, litigation and research cost (Schmaile and Munkvold, 2009).

The global economic losses due to mycotoxins in animal industries have been estimated to be as much as several hundred million dollars per annum (CAST, 2003). The economic losses incurred by animal industries due to fungal and mycotoxin contamination of cereal grains and by-products in

North America and Asia has been reported by Schmaile and Munkvold (2009). Wu (2007) reported that the estimated economic losses in the US due to contamination of animal feed with fumonisins varied between 1 and 20 million dollars in a year with no *Fusarium* ear rot outbreak. When coupled with *Fusarium* ear rot outbreak the total losses were estimated to increase to US$31–46 million.

Measures taken for prevention, decontamination or detoxification of mycotoxins can, therefore, be of great significance for animal health and productivity. The strategies that are used to reduce the bioavailability of mycotoxins, however, should destroy, remove or detoxify mycotoxins in feed and feed ingredients. At the same time, these methods should have little effect on the nutritional value of feedstuffs, and application of these strategies must be economically feasible under practical conditions (Galvano *et al*., 2001; Kabak *et al*., 2006).

Several approaches have been investigated to combat the adverse effects of feed-borne mycotoxins. Dietary inclusion of the nutritionally inert adsorbents is one of the most practical approaches (Galvano *et al*., 2001). The use of mycotoxin adsorbents can be an effective strategy to ameliorate mycotoxin-induced problems in animal production and health of livestock (Avantaggiato *et al*., 2007). Inorganic and organic adsorbents have been used to counteract the adverse effects of mycotoxins in livestock and poultry production. Inorganic adsorbents, including hydrated sodium calcium aluminosilicate (HSCAS), bentonites, zeolites and other clays, are the most broadly investigated compounds. Organic mycotoxin sorbents which are commonly used to reduce the bioavailability of mycotoxins in livestock and poultry diets include organic fibres (lignin), activated charcoal and yeast cell wall components.

The purpose of this chapter is, therefore to summarize the advances in strategies commonly used to prevent the fungal contamination and mycotoxin production, as well as the strategies applied to ameliorate the adverse effects of mycotoxins on performance and health of livestock and poultry.

13.2 Methods for decontaminating feedstuffs: strategies to prevent mycotoxin contamination pre- and post-harvest

The stages of intervention in preventing fungal and mycotoxin contamination of feedstuffs include (1) preventative measures taken before fungal infestation, (2) preventative measures taken during fungal invasion, and (3) intervention conducted whenever contamination of agricultural products has been detected (Jouany, 2007).

13.2.1 Prevention of plant infestation with fungi

The most effective control of mycotoxin contamination in feeds is prevention of fungal growth on plant products. The pre-harvest preventative

strategies include the development of plants that decrease the incidence of fungal infestation, restrict fungal growth and prevent toxin formation (Abdel-Wahhab and Kholif, 2008). Many agricultural practices can influence the crop contamination but even the best agricultural practices might not completely prevent mycotoxin contamination (Jouany, 2007). Hence, the most common approach used is prevention of fungal infestation of plants through treatment of field crops with fungicides. It should be noted, however, that significant species differences exist in the sensitivity of *Fusarium* species to fungicidal agents, and hence even the wide use of fungicides failed largely to prevent crop contamination of moulds, particularly the known toxin producer *F. graminearum* (Varga and Tóth, 2005).

Breeding for resistance, bio-competitive exclusion and genetic engineering
There are differences in the susceptibility of various plant species for *Fusarium* head blight which can be reflected in differences in the degree of mycotoxin contamination to which each species is susceptible (Kabak *et al*., 2006). Obtaining high levels of native genetic resistance to toxigenic fungi in various crop types has proven difficult. Development of resistant cultivars could be the ultimate solution in preventing the occurrence of deoxynivalenol (DON) and other mycotoxins (Trigo-Stockli *et al*., 2002). Resistant cultivars usually take significant time to develop and there are other associated problems. A barley variety which had been developed and improved for resistance against *Fusarium graminearum* had poor malting properties (Trigo-Stockli *et al*., 2002). Hence, breeding mycotoxin-resistant hybrids without sacrificing other desirable traits of the crops might never be achieved (Bailey *et al*., 2006). The use of non-toxigenic bio-competitive micro-organisms is the most recent approach used to reduce mycotoxin contamination of crops (Kabak and Dobson, 2009). Many non-toxigenic strains of *Aspergillus* fungi including *Aspergillus flavus* and *Aspergillus parasiticus* have been used to reduce AF contamination of cereal grains. These strains must be applied at an appropriate time and in a way that facilitates the successful competition with toxigenic AF producers (Kabak and Dobson, 2009). Transgenic approaches have been developed as an additional strategy to prevent mycotoxin biosynthesis or detoxify mycotoxins in the crop. Some of the fungi, including *Rhizopus stolonifer* and *Aspergillus fumigatus*, could be used as a source of enzyme for the prevention of mycotoxin formation. *A. fumigatus* is a well-known invasive fungus associated with a series of respiratory diseases in animals and humans. Genes encoding for detoxifying enzymes, however, could also be incorporated into a plant genome to produce transgenic crops capable of degrading several mycotoxins (Kabak and Dobson, 2009). Higa-Nishiyama *et al*. (2005) studied *in planta* detoxification of ZEN by incorporating the gene ZHD101, a lactonohydrolase from the fungus *Clonostachys rosea* in rice leaves. Detoxification of ZEN occurred when incubated with protein extract of

first-generation leaves. Detoxification was also observed in the second generation of transgenic seeds (Higa-Nishiyama *et al*., 2005).

Reducing contamination through field management
Crop rotation, tillage, soil fertilizers, planting date, chemical and biological control of fungal infection, insect and weed control, organic farming and modeling of mycotoxin risk at the field level are some of the solutions that could be used for prevention of plant infestation by fungi and associated mycotoxin contamination pre-harvest (Jouany, 2007). Crop rotation is important in breaking the chain of production of infectious materials (Kabak *et al*., 2006).

Detoxification of mycotoxins can be achieved by removal or elimination of the contaminated feedstuffs or by inactivation of toxins through physical, chemical and biological methods (Kabak *et al*., 2006). Although it is recommended to prevent plant infestation by fungi in the field, contamination with fungi and mycotoxins is unavoidable under certain environmental conditions (Varga and Tóth, 2005). The common factors that influence the production of mycotoxins include water activity, temperature, gas composition, and the presence of chemical preservatives and microbial interactions (Varga and Tóth, 2005).

13.2.2 Harvest and post-harvest control of contamination

Jouany (2007) summarized already the important factors that contribute to the control of mycotoxins during harvest and post-harvest. These include physiological stage of the plant, temperature during storage, and humidity level before and during storage (Jones *et al*., 1981; Ono *et al*., 2002). Other post-harvest strategies which are important in prevention of mycotoxin contamination include drying along with use of natural and chemical agents and irradiation (Kabak *et al*., 2006). The final level of mycotoxin contamination might depend on the time of harvest. It has been reported that earlier harvesting may result in lower concentrations of mycotoxins. Moisture content of grains is the main limiting factor for fungal growth. A water activity greater than 0.65 is essential for active fungal metabolism. *Aspergillus* can grow at a lower water activity, whereas higher water activity is required for the growth of *Fusarium* fungi. Wet spots may form during storage, further promoting fungal growth. These can be controlled by combined cooling and drying along with proper ventilation (Jouany, 2007).

Physical methods of contamination control
Physical treatment of contaminated grains includes sorting, washing, dehulling, density segregation, thermal treatment, grain milling, ultrasound, irradiation and solvent extraction; these are some of the methods used to control mycotoxin contamination at the post-harvest stage (Kabak *et al*., 2006). The success of these different methods, however, depends on the

degree of mycotoxin contamination, mycotoxin distribution throughout the grain and the method utilized (Trigo-Stockli *et al*., 2002). Siwela *et al*. (2005) reported a reduction of 92% AF concentrations in dehulled maize compared to hulled maize. Mechanical dehulling resulted in a 60% reduction in fumonisin concentrations in contaminated maize (Fandohan *et al*., 2006). Heat treatment of AFs in contaminated feedstuffs resulted in partial destruction. The need for high temperatures and pressures for effective detoxification of contaminated foods and feeds, however, reduces the acceptability of heat treatment as a practical means for AF detoxification (Samarajeewa *et al*., 1990). The influence of heating temperature and duration on *Fusarium* mycotoxins including DON, nivalenol (NIV) and ZEN was investigated in barley (Yumbe-Guevara *et al*., 2003). It was reported that at 220°C the time required to decompose 90% of DON, NIV and ZEN was 25 minutes, whereas the decomposition time for purified standards varied (11, 10 and 85 minutes for DON, NIV and ZEN respectively). The common problems associated with thermal treatment of feedstuffs to inactivate the mycotoxins are alteration of nutritional and organoleptic properties of the foods associated with generation of toxic pyrolysates at higher temperatures. Washing of contaminated grains has been shown to be effective in reducing DON in wheat; however, under commercial conditions, washing may not be feasible due to the additional cost of drying (Trigo-Stockli *et al*., 2002). Separating infected and damaged maize grains from the sound grains can result in 40–80% reduction in aflatoxin concentrations (Fandohan *et al*., 2005). Sorting could be done manually or mechanically. The benefit of sorting is that it decreases mycotoxin concentrations to safe levels without the production of toxin metabolites or any effect on the nutritional value (Hell *et al*., 2010). Methods such as screening, kernel sizing, electronic colour sorting, hand sorting, and blanching followed by electronic colour sorting have been used to manage mycotoxin contamination of peanuts (Dorner, 2008). Reductions of 81% in aflatoxin concentrations and 85% in fumonisin concentrations in contaminated maize were observed after high-speed sorting (Pearson *et al*., 2004). Concentrations of mycotoxins in milling by-products indicate the physical location of mycotoxins in whole grains and also colonization of the fungi in the grain seed (Scudamore, 2008). The potential reduction of DON levels in wheat resulting from milling has been previously reviewed by Kushiro (2008).

Chemical methods of contamination control

Chemicals such as ammonium hydroxide and calcium hydroxide monomethylamine have been tested for their ability to detoxify mycotoxins including trichothecenes, ZEN and AFs (Schatzmayr *et al*., 2006). Other chemicals investigated include hydrochloric acid, hydrogen peroxide, bisulfite, chlorinating agents and formaldehyde (Kabak *et al*., 2006). In general, addition of organic acids to compound feeds facilitates prevention of the formation of additional mycotoxins. Some chemicals and herbal compounds

have been evaluated for their ability to prevent fungal growth, for example of *Aspergillus parasiticus* (Gowda *et al*., 2004). Chemicals with antifungal properties, including propionic acid, ammonia, copper sulfate and benzoic acid, inhibited the growth of *Aspergillus parasiticus* completely in the potato dextrose agar. Other chemicals which inhibited fungal growth up to 34–64% were urea, citric acid and sodium propionate. Complete inhibition of AF production was caused by propionic acid, sodium propionate, benzoic acid and ammonia. Partial inhibition of AF production (up to 96%) was observed with citric acid, urea and copper sulfate. Among the herbal compounds tested, clove oil was shown to completely inhibit both fungal growth and AF production followed by other compounds including turmeric, onion and garlic (Gowda *et al*., 2004). The challenges associated with chemical use include production of toxic degradation products or products with undesirable characteristics such as off-flavours and reduced nutritional value (Kabak *et al*., 2006).

Biological methods of contamination control

Food safety issues, deterioration of nutritional quality of feedstuffs, inefficacy and cost implications are the problems associated with physical and chemical methods for detoxification of mycotoxins (Kabak *et al*., 2006). The yeast *Trichosporon mycotoxinivorans* isolated from the hind gut of termites was found to detoxify OTA and ZEN (Molnar *et al*., 2004). *Trichosporon mycotoxinivorans* completely detoxified OTA to OT-α in a mineral solution containing 400 μg/L of OTA after 2.5 hours of incubation. This special strain of yeast was also able to deactivate completely ZEN (1 mg/L) after 24 hours when suspended in mineral solution (Molnar *et al*., 2004). No ZEN metabolites including α- and β-ZEN were detected after 24 hours of incubation of *Trichosporon mycotoxinivorans*. Microbes including *Eubacterium BBSH 797* and *Trichosporon mycotoxinivorans MTV 115* have been shown to deactivate trichothecenes and OT respectively (Schatzmayr *et al*., 2006). In the same study, the ability of *Trichosporon mycotoxinivorans MTV 115* to detoxify ZEN was also documented. Some bacteria, including *Flavobacterium aurantiacum* B-184, have been shown to degrade AF. The bright orange pigmentation associated with this bacterium, however, would likely limit its applicability for food and feed fermentations (Bata and Lasztity, 1999).

Treatments resulting in reductions of fungal and mycotoxin contamination of feedstuffs before feeding are often not feasible since they are labour and cost intensive (Döll and Dänicke, 2004).

13.3 Feed additives to prevent mycotoxin absorption from the gastrointestinal tract

Decontaminating or detoxifying agents could be used as feed additives which are supposed to adsorb mycotoxins under the pH, temperature and

moisture conditions of the gastrointestinal tract (GIT), thereby preventing the transfer of mycotoxins to target organs (Dänicke *et al*., 2002). The active ingredients of such compounds include clay minerals, yeast cell wall components and microorganisms (Döll and Dänicke, 2004).

13.3.1 Efficacy of inorganic mycotoxin adsorbents

Clays and zeolitic materials are a diverse and complex family of aluminosilicates with a variety of functional characteristics (Bailey *et al*., 1998). Zeolites are crystalline hydrated aluminosilicates of alkali and alkaline earth cations (Papaioannou *et al*., 2005). HSCAS is an adsorbent compound derived from natural zeolite and has been demonstrated to adsorb mycotoxins with high affinity (Ramos and Hernandez, 1997). Inclusion of this compound in feedstuffs contaminated with AFs has shown protective effects against aflatoxicosis in livestock and poultry. The efficacy of HSCAS against ZEN and OTA is limited and no efficacy is seen for trichothecenes (Ramos and Hernandez, 1997).

Supplementation of a natural zeolite, clinoptilolite, decreased the number of affected broiler chickens and the severity of lesions in liver and kidney due to the feeding of 2.5 mg AF/kg feed (Ortatatli and Oguz, 2001). HSCAS (0.5 and 1%) was not effective in preventing the reduced average daily gain of piglets fed naturally contaminated corn (15 mg DON/kg) incorporated at 72, 50 and 25% of the diet (Patterson and Young, 1993). Decreasing the inclusion levels of contaminated corn from 72 to 25% improved average daily gain. Dilution of the contaminated corn with uncontaminated corn was the only effective method seen to reduce the toxicity of DON (Patterson and Young, 1993). Inclusion of 0.25% HSCAS did not prevent the adverse effects of 2 mg OTA/kg diet on performance and serum chemistry of broiler chickens (Santin *et al*., 2002).

An experiment was conducted to evaluate the effects of 5% dietary zeolite in preventing adverse effects of AFB_1 in broiler chicken diets (Sova *et al*., 1991). Broiler chickens were fed a control diet, a control diet + 5% zeolite, a contaminated diet containing 2.5 mg AFB_1/kg feed, and an AFB_1 contaminated diet + 5% zeolite. Inclusion of 5% zeolite in diets contaminated with 2.5 mg AFB_1/kg feed did not protect the birds from heterophilia and lymphopenia after 12 days of feeding (Sova *et al*., 1991). Supplementing a contaminated diet (2.5 mg AFB_1/kg feed) with 5% zeolite also did not prevent the adverse effects of AFB_1 on histopathological lesions of the liver. Zeolite added to the control diet induced focal lesions in the liver. The authors concluded that zeolite itself was not harmless. It could be hypothesized that addition of 5% zeolite may not be beneficial in preventing the adverse of AFB_1 at a concentration of 2.5 mg/kg feed. HSCAS, when included in diets of Leghorn and broiler chicks at 0.5%, significantly diminished the adverse effects of 7.5 mg AFB_1/kg feed (Phillips *et al*., 1988). Montmorillonite (0.5%; NovaSil Plus) partially prevented the reduction in

performance and altered blood parameters resulting from the feeding of 4 mg AFB_1/kg diet to broilers for six weeks (Bailey *et al*., 2006).

Adsorbents including clay (montmorillonite) and zeolite (clinoptilolite) minerals have been shown to adsorb mycotoxins that contain a polar functional group, including AFs. Mineral adsorbents exert low binding efficacy towards mycotoxins containing less polar functional groups, including *Fusarium* mycotoxins (Tomasevic-Canovic *et al*., 2003). The polar functional groups are required for efficient chemisorptions on hydrophilic negatively charged mineral surfaces to occur (Papaioannou *et al*., 2005). Modification of the surface properties of these adsorbents and control of hydrophobicity, however, might increase the binding ability of clay and mineral adsorbents to less or non-polar mycotoxins, including *Fusarium* mycotoxins. Modified zeolites (organo-zeolites) with different amounts of octadecyldimethylbenzyl-ammonium chloride and dioctadecyldimethyl ammonium chloride were tested for their ability to bind AFB_1, OTA, DON, ZEN and the ergopeptine alkaloids *in vitro* (Tomasevic-Canovic *et al*., 2003). Modified zeolites bound all the tested mycotoxins including AFB_1, OTA, ZEN and ergopeptine alkaloids, but not the trichothecene DON.

Dietary inclusion of organophilic modified montmorillonite did not prevent the adverse effects caused by *Fusarium* mycotoxins including DON (2.3–3.4 mg/kg diet) and ZEN (0.30–0.71 mg/kg diet) on performance of piglets in two different experiments (Döll *et al*., 2005). Biliary concentrations of ZEN and its metabolites were not different in piglets fed diets contaminated with *Fusarium* mycotoxins with or without addition of organophilic modified montmorillonite. This indicates that the aluminosilicate was not effective in binding ZEN in the gut. There were no differences in the serum concentrations of DON in the piglets fed the contaminated diet supplemented with or without aluminosilicate (Döll *et al*., 2005). Aluminosilicate was not effective in preventing the adsorption of *Fusarium* mycotoxins from the gastrointestinal tract (GIT) of piglets. The possible reason for inefficacy of aluminosilicate towards *Fusarium* mycotoxins is the non-polar nature of the mycotoxins. Clay minerals have been used in animal nutrition to bind mycotoxins, but the binders are more effective against AFs and less effective against other toxins (Schatzmayr *et al*., 2006).

It has been reported that spent canola oil bleaching clays prevented the depression of body weight and feed consumption caused by 3 mg T-2 toxin/kg feed in rats (Smith, 1984). Inclusion of defatted clay resulted in similar effects on the body weight and feed intake, whereas the rats fed T-2 toxin and residual oil showed very little effect on the measured parameters. Supplementation of bentonite to T-2 toxin contaminated diets, however, resulted in the prevention of adverse effects of T-2 toxin on body weight and feed intake of rats (Smith, 1984). The addition of spent canola oil bleaching clays to rat diets did not significantly promote the excretion of T-2 toxin in urine and feces. The differences in effectiveness of these clays in overcoming the T-2 toxicosis might not be related to the ion exchange capacity. The

effectiveness could be attributable to the gel-forming properties of these compounds when mixed with water and such gels may prevent adverse effects of the toxin on the mucosal surface of the GIT (Smith, 1984).

Dietary bentonite at concentration of 5% prevented the growth depression and feed refusal caused by feeding 3 mg T-2 toxin/kg diet to rats for two weeks (Carson and Smith, 1983a). A subsequent study conducted with different dietary concentrations of bentonite including 0, 2.5, 5, 7.5 and 10% showed that 10% bentonite was the most effective treatment in preventing the growth depression and feed refusal caused due to the feeding of T-2 toxin in rats. Fecal excretion of tritiated T-2 toxin was increased in rats fed 5, 7.5 and 10% bentonite. There was no effect of the feeding of bentonite on the excretion of tritiated T-2 toxin in urine. Increased concentration of tritiated T-2 toxin in digesta of the small intestine and in intestinal tissue was observed when rats were fed 5% bentonite (Carson and Smith, 1983a). The lack of a similar effect in rats fed higher concentrations of bentonite may be due to decreased intestinal transit time. This study proved the ability of bentonite in preventing the adverse effects of T-2 toxin by reducing intestinal absorption and by increasing fecal excretion of the toxin.

Diatomaceous earth is a naturally occurring sedimentary mineral silicate which is formed from the microscopic skeletal remains of diatoms. Addition of diatomaceous earth to diets containing 1 mg AFB_1/kg diet significantly prevented the adverse effects caused by AFB_1 in broiler chickens (Modirsanei *et al*., 2008).

An *in vitro* binding assay was conducted to test the efficacy of a carbon and aluminosilicate based mycotoxin adsorbent utilizing simulated *in vivo* conditions of the porcine GIT in multi-mycotoxin contaminated diets (Avantaggiato *et al*., 2007). Addition of up to 2% of the carbon and aluminosilicate based product prevented the absorption of mycotoxins including 88% AFB_1, 44% ZEN, 29% fumonisins and OT. The product was not effective, however, in reducing DON absorption (Avantaggiato *et al*., 2007). *In vitro* and *in vivo* analyses were conducted to determine the AFB_1 binding ability of calcium montmorillonite clay (NovaSil Plus) and prevention of the onset of aflatoxicoses and vitamin A depletion in broiler chickens (Pimpukdee *et al*., 2004). Isothermal analyses conducted between AFB_1 and NovaSil Plus to investigate and characterize critical sorption properties at equilibrium indicated that AFB_1 was bound tightly onto the surface of NovaSil Plus with high capacity and affinity. The chemisorption process was explained as exothermic and spontaneous. Supplementation of NovaSil Plus (0.5, 0.25 and 0.125%) in the AFB_1 contaminated diet protected broiler chickens from the effects of feeding 5 mg AFB_1/kg diet and prevented altered liver vitamin A concentrations even at lower dietary concentrations (Pimpukdee *et al*., 2004).

Addition of 0.25 or 0.375% HSCAS (T-Bind) to diets containing 5 mg AFB_1/kg and 8 mg T-2 toxin/kg prevented the adverse effects of AFB_1 on performance and relative organ weights of young broiler chickens (Kubena

et al., 1998). There was no effect of inclusion of HSCAS against the adverse effects of T-2 toxin. Supplementation of 0.8% HSCAS to diets containing 8 mg T-2 toxin/kg diet did not prevent oral lesions in broiler chickens. These results indicate that HSCAS was beneficial against AF, but not T-2 toxin, in broiler chickens.

Evaluation of an acidic phyllosilicate clay, a neutral phyllosilicate clay (HSCAS), and a common zeolite (clinoptilolite) to prevent the adverse effects of cyclopiazonic acid indicated that the supplemental inorganic adsorbents were not effective in preventing the adverse effects on performance and organ morphology (Dwyer *et al*., 1997). There was only a minor prevention of altered blood parameters.

Three inorganic adsorbents (0.5%) were tested for their efficacies in preventing the adverse effects of feeding 5 mg AFB_1/kg diet and 8 mg T-2 toxin/kg diet to broiler chickens (Bailey *et al*., 1998). These inorganic adsorbents were shown to have binding efficacies for AFB_1 and T-2 toxin *in vitro*. Inclusion of these adsorbents showed no beneficial effects in preventing the adverse effects of T-2 toxin in broiler chickens. Supplementation of one of the adsorbents to AF contaminated diets fed to broiler chickens resulted in prevention of adverse effects of AF on performance while the other two were not effective even against AF (Bailey *et al*., 1998).

The mechanism by which inorganic adsorbents including HSCAS prevent the absorption of AFs is through formation of a stable complex between HSCAS and AFs. This complex is not able to cross the luminal membrane of the GIT and thereby reduces the bioavailability of AFs (Ramos and Hernandez, 1997). More precisely, the mechanism is formation of a stable complex between the β-carbonyl system of the AF and an uncoordinated edge site of aluminium ions in HSCAS (Phillips, 1999). Pore sizes of the inorganic absorbents vary among adsorbents. Sources of inorganic adsorbents also influence the adsorption capacity comparing natural and synthetic sources (Galvano *et al*., 2001). The binding strength depends on the compatibility between pore size of the adsorbents and the mycotoxin structure.

13.3.2 Efficacy of organic mycotoxin adsorbents

Activated charcoal or carbon is a product obtained from pyrolysis of organic materials and is highly porous, insoluble powders having a high surface to mass ratio (Huwig *et al*., 2001). The adsorbing properties of activated charcoal depend on the source of material used to produce it. Physicochemical parameters including pore size, distribution and surface area are the other important factors contributing to the adsorption capacity of activated charcoal (Galvano *et al*., 2001). Activated charcoal has shown considerable promise in preventing aflatoxicosis (Edrington *et al*., 1997). Dietary inclusion of activated charcoal up to 10,000 mg/kg to diets containing 6.7% and 2% fat, and 4 mg OTA/kg, did not reduce OTA toxicity in broiler chickens (Rotter *et al*., 1989). *In vitro* binding studies indicated that when 50 mg of

activated charcoal was added to 10 mL of citrate–phosphate buffer at pH 7 containing 150 μg OTA, about 90% of OTA was adsorbed. The *in vitro* binding ability was reduced to 66% when a small amount of broiler chick feed was added to the mixture. Further addition of 50 mg activated charcoal increased the adsorption by 11% (Rotter *et al*., 1989).

Inclusion of 1% activated charcoal to diets containing 30 mg fumonisin B_1 (FB_1)/kg diet from culture material did not prevent the adverse effects of FB_1 on performance, biochemical parameters and organ pathology of weanling piglets (Piva *et al*., 2005). The ineffectiveness of activated charcoal in pigs could be due to limited exposure of the pores to FB_1 or limited substrate specificity of activated charcoal. Ingestion of FB_1 contaminated diet supplemented with activated charcoal increased the severity of effects of FB_1 (Piva *et al*., 2005). These changes indicate the possibilities that activated charcoal could bind micronutrients including vitamins, minerals and amino acids which are essential for growth and health of pigs. It has been previously reported that FB_1 can absorb folate and also inhibit intestinal folate transporters. Thus FB_1 could enhance the dietary requirements for folic acid in pigs (Piva *et al*., 2005). Management problems associated with inclusion of activated charcoal in mycotoxin contaminated diets may include blackening of feed, animals and their environment and loss of nutrients, as activated charcoal binds a broad variety of organic substances, including certain amino acids and many vitamins and hence can be given only for a short period of time.

Organic adsorbents including lignin-rich alfalfa fibre were effective in ameliorating the toxicity of T-2 toxin (Carson and Smith, 1983b) and ZEN (James and Smith, 1982; Stangroom and Smith, 1984). The alfalfa fibre might not cause a major dilution of the nutrient density but inclusion rates for efficacy are high, and this is impractical with respect to animal and poultry diets. Addition of 1% micronized wheat fibres to OTA contaminated diets significantly prevented adverse effects on weights of kidney and liver in piglets (Aoudia *et al*., 2009). Supplementation of micronized wheat fibres also protected piglets in terms of OTA accumulation in plasma, kidney and liver by decreasing bioavailability (Aoudia *et al*., 2009). The mechanism by which dietary fibres exert their protective effects in the GIT is by adsorbing xenobiotics and hence lowering intestinal absorption (Sera *et al*., 2005).

A polymeric glucomannan mycotoxin adsorbent (GMA) derived from the cell wall of yeast has been shown to prevent some of the deleterious effects of fungal toxins on performance and metabolism of livestock and poultry. The β-D-glucan fraction of the yeast cell wall is involved in the binding process of mycotoxins, and the structural organization of β-D-glucans modulates binding strength (Yiannikouris *et al*., 2004a, b).

Supplementation of GMA (0.2%) to diets high in naturally contaminated grains (9.7 mg/kg DON, 21.6 mg/kg fusaric acid and 0.8 mg/kg ZEN) prevented *Fusarium* mycotoxin-induced elevation in red blood cell counts, hemoglobulin and serum uric acid concentrations in broiler chickens

(Swamy *et al*., 2002a). Supplementation of GMA to the diet with higher concentration of contaminated grains also prevented *Fusarium* mycotoxin-induced decrease in biliary IgA concentrations. In yet another study, supplementation of GMA to the contaminated diet prevented the reduction in B-cell counts caused by feeding of a high concentration of contaminated grains (9.5 mg/kg DON, 21.4 mg/kg fusaric acid, 0.7 mg/kg ZEN and 0.5 mg/kg 15-acetyl-DON; Swamy *et al*., 2004). GMA supplementation to the contaminated diet, however, failed to prevent the adverse caused by *Fusarium* mycotoxins on performance of broiler chickens (Swamy *et al*., 2002a, 2004). Girish and Devegowda (2006) reported the preventative effects of GMA (0.1%) on performance, organ weights and immune parameters of broiler chickens when included in diets containing 2 mg AF/kg and 1 mg/kg T-2 toxin.

GMA supplementation to diets naturally contaminated with *Fusarium* mycotoxins ameliorated the adverse effects on turkey blood cell counts including basophils and monocytes (Chowdhury *et al*., 2005a). The feeding of GMA also prevented the adverse effects of feed-borne *Fusarium* mycotoxins on turkey performance during starter, developer and finisher phases (Chowdhury and Smith, 2007). Inclusion of GMA to contaminated diets (2.2–3.3 mg DON/kg diet) prevented the adverse effects of *Fusarium* mycotoxins on body weight gains of turkeys during grower and developer phases (Girish *et al*., 2008a). GMA supplementation prevented the elevated levels of uric acid concentrations after 12 weeks of feeding. Supplementation of the contaminated diet with GMA also prevented decreases in contact hypersensitivity response to dinitrochlorobenzene after 24 hours compared to birds fed diets contaminated with *Fusarium* mycotoxins (Girish *et al*., 2008a). Girish and Smith (2008) observed the preventative effects of GMA on small intestinal morphology of turkeys when supplemented to *Fusarium* mycotoxin contaminated diets. At the end of the starter phase, supplementation of the contaminated diet with GMA prevented decreased duodenal villus height. Supplementation with GMA also prevented the reduction in jejunal villus height and apparent villus surface area and ileal submucosa thickness caused by the feeding of contaminated diets to turkeys for six weeks (Girish and Smith, 2008). The preventative effect of GMA on *Fusarium* mycotoxin induced pons serotonergic turnover in turkey brain has been documented by Girish *et al*. (2008b). Addition of GMA to the contaminated diet prevented the decrease in pons concentrations of 5-hydroxyindoleacetic acid and the ratio of 5-hydroxyindoleacetic acid to serotonin.

GMA supplementation of laying hen diets naturally contaminated with *Fusarium* mycotoxins prevented adverse effects on the total number of B-lymphocytes in the peripheral blood and biliary IgA concentration compared to birds fed unsupplemented diets (Chowdhury *et al*., 2005b). Chowdhury and Smith (2004) reported the beneficial effects of feeding GMA to laying hens exposed to diets naturally contaminated with *Fusarium* mycotoxins. The feeding of GMA restored feed intake to control levels and

improved feed intake/egg mass. Supplemental GMA prevented mycotoxin-induced renal enlargement. Addition of GMA to the contaminated diets prevented mycotoxin-induced reductions in egg production, egg mass, yolk weight and egg shell weight (Chowdhury and Smith, 2004). GMA supplementation also restored plasma concentrations of glucose and uric acid and amylase activity. GMA supplementation to broiler breeder hen diets naturally contaminated with *Fusarium* mycotoxins including 12.6 mg DON/kg feed prevented decreased egg shell thickness after four weeks of feeding and early embryonic mortality (Yegani *et al*., 2006a). Supplementation with GMA also prevented a reduction in the antibody titres against infectious bronchitis virus. Yegani *et al*. (2006b) also observed increased concentrations of serotonin and decreased 5-hydroxyindoleacetic acid to serotonin concentration ratios in the pons regions in the brains of laying hens fed *Fusarium* mycotoxin-contaminated diets, and GMA supplementation prevented this effect.

An experiment was conducted with pigs to evaluate the efficacy of GMA (0.05, 0.1 and 0.2%) in preventing the adverse effects of feed-borne *Fusarium* mycotoxins. Diets contained contaminated grains averaged 5.5 mg DON/kg, 0.5 mg 15-acetyl-DON, 26.8 mg/kg fusaric acid and 0.4 mg/kg ZEN (Swamy *et al*., 2002b). The inclusion of 0.05% GM polymer prevented the elevated dihydroxyphenyl acetic acid concentrations in pons caused by the feeding of contaminated diets (Swamy *et al*., 2002b). Supplementation of 0.05 and 0.1% GMA in the contaminated diet prevented increases in serum IgA concentrations, whereas 0.1% GMA was effective in preventing elevated serum IgM concentrations. Addition of 0.2% GMA to the contaminated diet prevented the reduction in mean corpuscular hemoglobulin, and GMA supplementation at 0.05% prevented a decrease in the liver weights (Swamy *et al*., 2002b). Feeding gestating sows diets naturally contaminated with *Fusarium* mycotoxins can increase the incidence of stillborn piglets and this effect can be reduced by dietary GMA supplementation (Diaz-Llano and Smith, 2006). Supplementing contaminated feed with GMA counteracted the reduction in serum protein and serum urea levels observed in lactating sows fed contaminated feed (Diaz-Llano and Smith, 2007).

Dietary GMA decreased the liver lesions in pigs fed 968 μg AFB_1/kg feed. There was no effect of GMA, however, on impaired phase I liver enzymes due to both AFB_1 (482–1912 μg/kg feed) and T-2 toxin (593–2067 μg/kg feed; Meissonnier *et al*., 2009). Supplementation with GMA prevented delayed ovalbumin specific lymphocyte proliferation in pigs fed AFB_1 (482–1912 μg/kg feed). Inclusion of GMA also prevented a reduction in antibody production against ovalbumin in pigs fed T-2 toxin at 1155–2067 μg/kg feed (Meissonnier *et al*., 2009). Dänicke *et al*. (2007) reported that supplementation of GMA was not effective in preventing the adverse effects caused by the feeding of *Fusarium* mycotoxin contaminated diets to piglets. There was a lack of interaction between the

mycotoxin-contaminated diet and GMA on performance and in adsorbing the DON from the GIT (Dänicke *et al*., 2007).

A total mixed ration containing a blend of feedstuffs naturally contaminated with *Fusarium* mycotoxins containing DON (3.6 g/g of dry matter) as the major contaminant was fed for 56 days to 18 mid-lactation Holstein cows (Korosteleva *et al*., 2007). GMA supplementation prevented alterations in total serum protein and globulin levels, and serum urea and IgA concentrations compared to those fed unsupplemented contaminated diets.

Supplementing a contaminated diet with GMA partially prevented the adverse effects of *Fusarium* mycotoxins on feed intake of horses (Raymond *et al*., 2003). Supplementation of GMA also prevented an increase in serum gamma-glutamyl-transferase (GGT) concentrations when contaminated grain was fed to non-exercising, light, mixed-bred mares for 21 days. Raymond *et al*. (2005) conducted the study on the effects of feed-borne *Fusarium* mycotoxins on feed intake, metabolism and indices of athletic performance of exercised horses. In exercised horses supplementing the contaminated diet with 0.2% GMA did not prevent reduced feed intake or weight loss caused by the feeding of a diet contaminated with *Fusarium* mycotoxins.

Non-digestible adsorbents may adsorb mycotoxins in the lumen of the GIT (Bata and Lasztity, 1999). It has been reported that yeast cell wall components (polysaccharides: glucan and mannan, proteins and lipids) possess many adsorption sites. Adsorption mechanisms include hydrogen bonding and ionic or hydrophobic interaction (Huwig *et al*., 2001). Feed-borne yeast cell wall components are capable of adsorbing mycotoxins in the GIT, thereby limiting their bioavailability (Yiannikouris *et al*., 2004a). Cell walls from different strains of yeast including *Saccharomyces cerevisiae* have been tested for their ability to adsorb ZEN using Hill's model (Yiannikouris *et al*., 2004a). Cell walls of yeast strains with higher levels of β-D-glucans show higher absorptive capacity with higher association constants and higher affinity rates than those with lower proportions of β-D-glucans (Yiannikouris *et al*., 2004a). Cooperativity and three-dimensional structures of β-D-glucans indicate that the weak non-covalent bonds are involved in the complex-forming mechanisms associated with ZEN. The hydroxyl, lactone and ketone groups of the ZEN molecule, and the hydroxyl groups of the (1–3)-β-D-glucan single helix, are involved in hydrogen bonding, whereas a van der Waals stacking interaction occurs between the phenyl moiety of ZEN and the two opposite β-D-glucopyranose moieties of (1–3)-β-D-glucans (Yiannikouris *et al*., 2004c). (1–6)-β-D-glucan side chains also exhibit stabilizing effects by increasing the van der Waals interactions with ZEN. Chains of (1–3)-β-D-glucans are organized in helical structures of six β-D-glucopyranose units per turn of the helix. This structure can form highly relaxed triple-helix and/or single-helix organizations depending on environmental conditions (Yiannikouris *et al*., 2004c). It has also been reported that the hydroxyl, ketone and lactone groups are

involved in the formation of both hydrogen bonds and van der Waals interactions between DON, AFB_1 and patulin, and β-D-glucans. In conclusion, differences in the binding capacity of individual mycotoxins are due to their specific physical and chemical characteristics. The stereochemistry and hydrophobic nature of mycotoxins may be important and can contribute to the differences in their affinity for β-D-glucans. β-D-glucans can interact with a wide range of mycotoxins, and this situation is commonly encountered under field conditions (Yiannikouris *et al*., 2006).

13.3.3 Mycotoxin inactivators (enzymes, probiotics and prebiotics)

An alternative approach to prevent mycotoxicoses is the use of microorganisms capable of inactivating mycotoxins through enzymatic modification (Bata and Lasztity, 1999). These microbes are, therefore, categorized as mycotoxin-deactivating feed additives. Degradation is generally specific, irreversible and an environmentally friendly mechanism of detoxification (Politis *et al*., 2005).

The feeding of probiotic *Eubacterium* sp. to chickens counteracted the adverse effects caused by dietary DON on intestinal glucose transport (Awad *et al*., 2009). *Eubacterium* sp. converts DON into the less toxic De-epoxy DON. In turn, the impaired nutrient transport is restored. When broilers were fed inulin as a prebiotic, increased glucose absorption was seen in the jejunum and colon when birds were pre-treated with 10 μg DON/mL (Awad *et al*., 2009). This could be attributed to the selective promotion of specific bacterial strains including bifidobacteria and lactobacilli through the addition of inulin. These bacteria produce short-chain fatty acids (SCFA) and an increase in blood SCFA might increase serum glucose transporters, leading to increased glucose transport even in the presence of DON (Awad *et al*., 2009). The addition of probiotic *Eubacterium* to a diet containing 10 mg DON/kg feed alleviated the intestinal histomorphometric alteration caused by DON in broiler chickens (Awad *et al*., 2006).

Trichosporon mycotoxinivorans, a novel strain of *Trichosporon*, was evaluated for its ability to counteract the adverse effects of OTA on immune parameters of broiler chicks (Politis *et al*., 2005). Dietary supplementation of *Trichosporon mycotoxinivorans* at concentrations of 10^4, 10^5 and 10^6 CFU/g feed contaminated with OTA (0.5 mg/kg feed) significantly prevented decreased cell viability and total cells associated and membrane bound urokinase-plasminogen activator of macrophages and heterophils (Politis *et al*., 2005).

Dietary application of enzymes, such as PVPP, a polyvinylpolypyrrolidone basic structure that carries epoxidase and lactonase, prevented the decrease of peripheral T-lymphocyte counts in broiler chickens caused by feeding 2.5 mg AFB_1/kg diet (Celik *et al*., 2000). In contrast, inclusion of graded levels of these enzymes to *Fusarium* mycotoxin (DON and ZEA) contaminated diets failed to counteract the adverse effects on performance,

metabolism and immunity of broiler chickens (Dänicke *et al*., 2003). The additive also had no influence on nutrient digestibility and concentrations of ZEN and its metabolites in excreta. Supplementation to the diets containing 2.7–2.8 mg DON/kg feed was not effective in preventing the adverse effects on performance of pigs (Dänicke *et al*., 2004). There was no effect of addition of Mycofix Plus to *Fusarium* mycotoxin contaminated diets on performance and production of laying hens (Danicke *et al*., 2002). There was no interaction between the contaminated diet and Mycofix Plus on nutrient digestibility and metabolizability of gross energy; some mycotoxin-independent effects of the detoxifying agent were observed (Danicke *et al*., 2002).

The protective effects of a commercial formulation containing an encapsulated bacterium (Mycofix) against the adverse effects of 4,15-diacetoxyscirpenol (DAS) in broiler chickens were determined (Diaz, 2002). Supplementation at a level of 0.75 and 1.5 kg/ton of feed prevented the adverse effects caused by the feeding of 1 mg DAS/kg feed, while partial protection was observed against the toxic effects resulting from the feeding of 2 mg DAS/kg feed. Supplementation at a level of 2 kg/ton of feed showed partial and non-significant protection against the immunotoxic effects of OTA (0.25 mg/kg) and T-2 toxin (0.5 mg/kg) when fed in combination to broiler chickens (Xue *et al*., 2010). Toxic effects of OTA (0.5 and 1 mg/kg) and the preventative effects of a yeast-product derived from *Trichosporon mycotoxinivorans* (Mycofix Plus) on the performance, serum enzymes and organ clinico-pathomorphology were studied in broiler chickens at 42 days (Hanif *et al*., 2008). Supplementation at a level of 1–2 kg/ton prevented the negative effects of OTA on feed conversion ratio, liver enzymes, organ pathomorphological and histological alterations.

13.3.4 Advantages and disadvantages of mycotoxin detoxifying and decontaminating feed additives

Due to low inclusion rates and easy management of mycotoxin-inactivating additives, an increased acceptance of these products by the livestock and poultry industries has led to the introduction of a variety of diverse materials labeled as feed-borne mycotoxin inactivators (Marroquin-Cardona *et al*., 2009). An important factor to be taken into account while choosing a potential mycotoxin inactivator is the inclusion rate for efficacy. The efficacy of mycotoxin inactivators, however, will vary for different mycotoxins. The safety of these commercially available products should undergo thorough testing through bioassays. Experimentation with naturally contaminated grains reflects practical field conditions under which such agents will be utilized. Experimentation with purified crystalline mycotoxins is less reflective of commercial reality (Döll and Dänicke, 2004).

Clay minerals have been described as being only specific for AFs but not for other mycotoxins including *Fusarium* mycotoxins (Schatzmayr *et al*.,

2006). The possible reasons for this could be due to the less polar nature of *Fusarium* mycotoxins. It has also been shown that clays can adsorb micronutrients and have negative effects in reducing bioavailability of minerals and other trace elements (Moshtaghian *et al*., 1991). The other risk associated with the use of natural clays as feed additives could be their contamination with dioxins (Jouany, 2007). Organic binders, including polymeric glucomannan mycotoxin adsorbents, may be more efficient against a wide range of mycotoxins compared to inorganic binders and this could be beneficial when feed is contaminated with more than one mycotoxin. Organic binders are degradable and hence they are ecofriendly, whereas inorganic adsorbents accumulate in the environment due to their non-biodegradable nature and this can lead to pollution of soils and pastures (Jouany, 2007). Activated charcoal, moreover, is a relatively non-specific adsorbent and might bind to essential nutrients at higher levels of dietary inclusion (Huwig *et al*., 2001). Mycotoxin inactivators (enzymes) are specific for each mycotoxin. Supplementation of mycotoxin inactivators as feed additive may not be beneficial under circumstances when feed will be contaminated with more than one mycotoxin.

13.4 Effects of nutritional supplementation on mycotoxicoses

Deficiencies of nutritional components might enhance susceptibility of farm animals to the toxic effects of feed-borne mycotoxins. Apart from the more practical approach of using mycotoxin adsorbents and inactivators to counteract mycotoxicoses in livestock and poultry, one of the most acceptable methods would be nutrient supplementation.

Various metabolic pathways related to growth and immunity are regulated through antioxidant and pro-oxidant balance in the body (Yu *et al*., 2009). Alterations caused in this balance could lead to reduced growth and altered immunity. Many of the *Fusarium* mycotoxins, including T-2 toxin and fumonisins, and *Aspergillus* toxins are known to cause cell membrane damage through enhanced lipid peroxidation. One of the mechanisms of cell toxicity of mycotoxins is mediated through production of reactive oxygen species and free radicals (Atroshi *et al*., 2002). Inactivation of free radicals could be an effective method to inhibit such oxidation. Chain breaking antioxidant compounds are effective against these mycotoxins because of their ability to neutralize superoxide anions, thereby protecting cell membranes from mycotoxin-induced damage (Galvano *et al*., 2001). Antioxidant compounds including vitamins E, A and C, provitamins, carotenoids, chlorophyll and its derivatives, and selenium are commonly used to counteract the adverse effects of mycotoxins. Synthetic antioxidant compounds, including butylated hydroxytoulene and butylated hydroxyanisole, can also be effective in protecting against the toxic effects of mycotoxins (Atroshi *et al*., 2002).

Yu *et al*. (2009) evaluated the protective effects of selenium and vitamin E in rats fed maize naturally contaminated with mycotoxins. Supplementation of naturally contaminated maize with antioxidative additives, including selenium-enriched yeast and vitamin E, prevented oxidative stress and alteration in the liver enzyme profiles in rats. Supplementation with GMA to diets contaminated with T-2 toxin (8.1 mg/kg feed) prevented partially adverse effects on chicken liver antioxidant status, including the concentrations of selenium, alpha-tocopherol, glutathione, total carotenoids, ascorbic acid and selenium-dependent glutathione peroxidase (Dvorska *et al*., 2007). Supplementation of chicken diets with GMA partially protected lipid peroxidation in liver, whereas supplementation with combinations of organic selenium and GMA provided further protection against antioxidant depletion and lipid peroxidation (Dvorska *et al*., 2007). Mycotoxins can be detoxified in the liver through the glutathione system. Glutathione contains cystine, a derivative of methionine, and hence the metabolic concentrations of methionine may be depleted, leading to decreased performance during mycotoxicoses (Faixova *et al*., 2007). Supplementation of essential amino acids, including methionine and antioxidant components including selenium and vitamins, may, therefore, be beneficial during mycotoxicoses.

Supplementing broiler diets containing 3 mg DON/kg feed with selenium-enriched yeast (1.4 mg/kg feed) prevented most of the adverse effects of DON on plasma constituents, including concentrations of calcium and magnesium, and alanine aminotransferase activity (Faixova *et al*., 2007). There was no effect of the dietary selenium-enriched yeast, however, on feed-borne DON-induced alterations in concentrations of chloride, total proteins, triglycerides and free glycerol.

Increasing dietary crude protein from 18 to 20% prevented the adverse effects caused by feeding of 132 μg AFB_1/kg diet to pigs (Coffey *et al*., 1989). Incremental fat (5%) supplementation improved the efficiency of feed utilization in the pigs fed AFB_1 contaminated corn. Interactions between AF, crude protein and fat levels have been observed for plasma cholesterol concentration (Coffey *et al*., 1989). Inclusion of fat or increasing the crude protein level prevented plasma cholesterol reductions in pigs fed AFB_1. Supplementing swine diets containing 18% crude protein with 0.25% L-lysine HCl prevented decreased performance caused by feed-borne AFB_1. Supplementation of an 18% crude protein pig diet with 0.15% methionine, moreover, partially prevented reduced average daily gain compared to pigs fed 186 μg AFB_1/kg diet (Coffey *et al*., 1989). Manipulation of diet with precursor amino acids that control neurotransmitters and their competitors for active transport across membranes has potential in overcoming the feed refusal caused by *Fusarium* mycotoxins (Cavan *et al*., 1988).

Exposure of rats to fumonisin induced brain lesions, including increased sphinganine concentrations, hypomyelination and an impairment of 2,3-cyclic nucleotide 3-phosphohydrolase activity (CNP; Carratu *et al*., 2003).

Hypomyelination and other lesions induced by fumonisin, however, were comparable to lesions caused by early weaning of rats from dams resulting in a lack of nutrients for myelin synthesis. Hypomyelination and impaired CNP activity, therefore, could be at least partly due to nutritional deficiency caused by fumonisins. Carratu *et al*. (2003) reported the reduced uptake of folate by various cell lines treated with fumonisins. Thus the resulting brain lesions could be attributable to folate deficiency associated with fumonisin ingestion caused by depletion of sphingolipids. Incremental dietary folate supplementation would be beneficial in overcoming the adverse effects of fumonisins on rat brain function.

Dietary supplementation with yeast and amino acids (methionine and cysteine) reduced liver lesions in rats fed 400 μg AF/kg diet (Baptista *et al*., 2008). Melatonin prevented OTA (289 μg/kg per day) induced changes in rat lipid peroxidation, hepatic and blood activities of glutathione peroxidase, and blood activities of catalase and superoxide dismutase (Soyoz *et al*., 2004). These results indicate the significance of the antioxidant defence system, including the free radical scavenging action of the pineal hormone melatonin. The mechanism of action of melatonin in scavenging the free radicals is through donation of electrons to detoxify hydroxyl radicals (Soyoz *et al*., 2004). The antioxidant effects of vitamins A, C and E were tested in human lymphocytes treated with 5 μM AFB_1 (Alpsoy *et al*., 2009). Simultaneous supplementation with vitamins A, C and E prevented AFB_1 induced reductions in glutathione and activities of superoxide dismutase and glutathione peroxidase and the increase in concentration of malondialdehyde through inhibition of production of reactive oxygen species (Alpsoy *et al*., 2009).

The mechanism by which vitamin E reduces reactive oxygen species and protects the polyunsaturated fatty acid portion of lipophilic membranes is through donation of its phenolic hydrogen to a chain propagating lipid peroxyl radical and producing the less reactive alpha-tocopheroxyl radical (Baldi *et al*., 2004; Jaradat *et al*., 2006). Vitamin A acts as a protecting and stabilizing agent of cell membranes. Baldi *et al*. (2004) suggested that location of all-*trans*-retinol within the membrane could play an important role in scavenging peroxyl radicals, hence stabilizing the membrane.

The proposed mechanism by which dietary protein or limiting amino acids protect against aflatoxicosis is through enhancing the activity of mixed function oxidase (Coffey *et al*., 1989). Mixed function oxidase is involved in detoxification of AFB_1 into less toxic metabolites. The glutathione system is also involved in the detoxification. Amino acids involved in the synthesis of glutathione include cysteine, glutamine and glycine. It has been reported that increased levels of methionine have shown to increase the liver glutathione levels in rats (Seligson and Rotruck, 1983). Increased levels of methionine might therefore increase glutathione concentrations (Coffey *et al*., 1989). Supplementation of limiting amino acids including lysine and methionine may be beneficial in preventing the adverse effects of AFB_1.

Addition of various antioxidants to feeds is often not practical due to the economic and labeling restrictions. All antioxidants may not be suitable for all feeds due to problems associated with solubility, flavours and colour and interaction with feed components (Atroshi *et al*., 2002).

13.5 Conclusions and implications

Classification and evaluation of mycotoxin sequestering and detoxification agents is complex. Care must be taken while making any conclusions for a given adsorbent group, as all commercially available products in a given classification will not always have the same composition or efficacy (Diaz and Smith, 2005). The limitations associated with inorganic adsorbents and other methods of decontamination of mycotoxins point to the use of organic adsorbents from a practical standpoint. Suitable organic adsorbents which satisfy the commercial desirability of low inclusion rates, multiple mycotoxin binding ability, economic feasibility, biodegradable properties and no innate toxicity may be the best choice to reduce the economic losses due to feed-borne mycotoxins in the animal industries. Combinations of mycotoxin-deactivating enzymes may be beneficial if they have the ability to degrade multiple mycotoxins in feed.

13.6 Future trends

The most practical approach to minimizing the adverse effects of mycotoxins in livestock and poultry diets is by the use of mycotoxin adsorbents. Development of biodegradable mycotoxin adsorbents with low inclusion rates – economically feasible, broad spectrum, no residues in animal products and no effect on dietary nutritional composition – could be the optimal future approach to counteracting mycotoxicoses. Mycotoxin-deactivating enzymes are specific in nature and are capable of degrading individual mycotoxins. Identification and isolation of combinations of enzymes capable of degrading multiple mycotoxins may also be a future practical approach to combat mycotoxin-induced adverse effects.

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14

Dietary exposure of livestock and humans to hepatotoxic natural products

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Abstract: Hepatotoxicity is a multi-factorial and multi-consequential assault on the liver by various toxins including many natural toxins that may be present in animal feed and human food. Some examples of hepatotoxins are given but the main emphasis is focused on the hepatotoxic dehydropyrrolizidine alkaloids. The chemical diversity of the dehydropyrrolizidine alkaloids and their complex mechanism of action following acute or chronic exposure provide challenges for analysis and toxicological evaluation.

Key words: natural hepatotoxins, livestock poisoning, human poisoning, pyrrolizidine alkaloids.

14.1 Introduction

In the context of this chapter, plant-associated bioactive natural products include those produced by plants *per se* (secondary plant metabolites) and also by those organisms that grow on or within the plant, such as endophytic and saprophytic fungi, bacteria and insects that are consumed along with the plant. The diversity of such plant-associated bioactivities and related potencies or efficacies is a manifestation of complex evolutionary interactions and provides a font of opportunity for researchers of various disciplines (various authors in Colegate and Molyneux, 1993, 2008). However, the beneficial impacts of plant-associated bioactive natural products are constrained by the level and rate of exposure (the ingested dose) and they therefore may exert toxic effects when such limits are exceeded. The specific bioactivities that are the focus of this chapter are those that have an adverse effect on the liver, the hepatotoxins.

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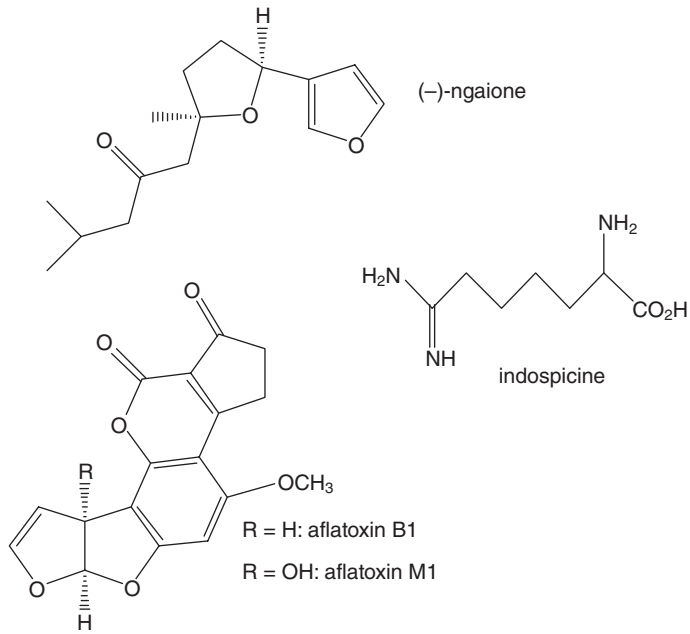


Fig. 14.1 Structures of the hepatotoxic (–)-ngaione, indospicine and aflatoxins B1 and M1.

It has been recognized for several decades that the higher incidence of early onset human liver disease, in developing countries in particular, is likely to be due to naturally occurring hepatotoxins, either as natural components or as contaminants of the food (Schoental, 1963). There are two main types of intoxication by the hepatotoxins, i.e. primary and secondary. The 'primary poisoning' type involves direct access to the hepatotoxin-producing organism that is a natural component of the food being consumed or is an inadvertent contaminant of otherwise healthy food. For example, (–)-ngaione (Fig. 14.1) is just one of a number of furano sesquiterpene pro-toxins, requiring hepatic activation for toxicity, that comprise the essential oils of plants such as *Myoporum tetrandrum* (boobialla) (Seawright *et al.*, 1982). It has been suggested that cattle are less susceptible than sheep (for example) to the toxic effects of these compounds, since there is a lower rate of bovine hepatic microsomal mixed function oxidase activity. This results in primarily a centrilobular necrosis in cattle but a periportal necrosis in sheep and other species with a higher microsomal mixed function oxidase activity (Allen *et al.*, 1978). The 'secondary poisoning' type of exposure is less common and involves ingestion of food derived from an animal previously exposed to the natural hepatotoxins, i.e. toxic residues. An example of secondary poisoning

is the extensive hepatic disease that dogs develop when they are fed indospicine (Fig. 14.1)-contaminated meat from horses that had grazed *Indigofera linnaei* (Hegarty *et al*., 1988). In some cases the primary hepatotoxin can be metabolized after ingestion and absorption and it is the metabolite that causes the secondary hepatotoxicity. For example, aflatoxin B1 (Fig. 14.1), produced by aspergilli (e.g. *A. flavus* and *A. parasiticus*) in improperly stored food and feed materials, can cause severe liver damage and has been classified as a human liver carcinogen. However, when present in the feed for dairy cows, this primary hepatotoxin is biotransformed in the liver to aflatoxin M1 (Fig. 14.1) which is subsequently excreted in milk. This then can cause 'secondary poisoning' of consumers of the milk and dairy products derived from the animals primarily exposed to the aflatoxin B1-contaminated feed (Sultana and Hanif, 2009).

Deleterious effects of natural products on the liver, or hepatotoxicity, can be due to many different types of chemical structure, with differing pathogeneses, pathology and consequences (for examples, see various authors in Colegate and Dorling, 1994; Zimmerman, 1999). Following the brief description of some examples of this hepatotoxic diversity, the major group of bioactive natural products discussed in this chapter is the hepatotoxic, pneumotoxic, carcinogenic and genotoxic dehydropyrrolizidine alkaloids (DHPAs) and their *N*-oxides.

14.2 The liver and hepatotoxicity

The liver has the critical role of maintaining a large portion of the body's metabolic and homeostasis systems, including processing of dietary amino acids, carbohydrates, lipids and vitamins; removal of microbes and toxins from the gastrointestinal blood; synthesis of plasma proteins; and detoxification and excretion of endogenous waste products and xenobiotics. Since the liver has a key role in energy production, protein metabolism and homeostasis, liver failure is quickly followed by failure of multiple organs with consequences that can include cardiovascular collapse, renal failure and death.

The liver is vulnerable to physical, metabolic, toxic, infectious, immunological and neoplastic insults. Though the insults vary, the hepatic response is limited to degeneration, necrosis or apoptosis, inflammation, regeneration and fibrosis. Therefore, only rarely do the various liver diseases produce specific or characteristic lesions. Additionally the liver enjoys an enormous functional reserve that often masks the clinical impact of early damage. Consequently, most hepatic diseases are insidious processes in which the symptoms or signs of decompensation occur over weeks, months and even years. Often, when clinical liver failure occurs, the typical lesions of the initiating cause are obscured by the massive fibrosis, degeneration, necrosis and regeneration that characterize chronic liver failure.

14.3 Types of adverse effect on the liver

14.3.1 Acute toxicity

Acute liver injury is often a result of cytotoxicity following exposure to relatively high doses of hepatotoxin for a short duration. Some of the most potent natural hepatotoxins are the cyclic peptide amatoxins (Fig. 14.2)

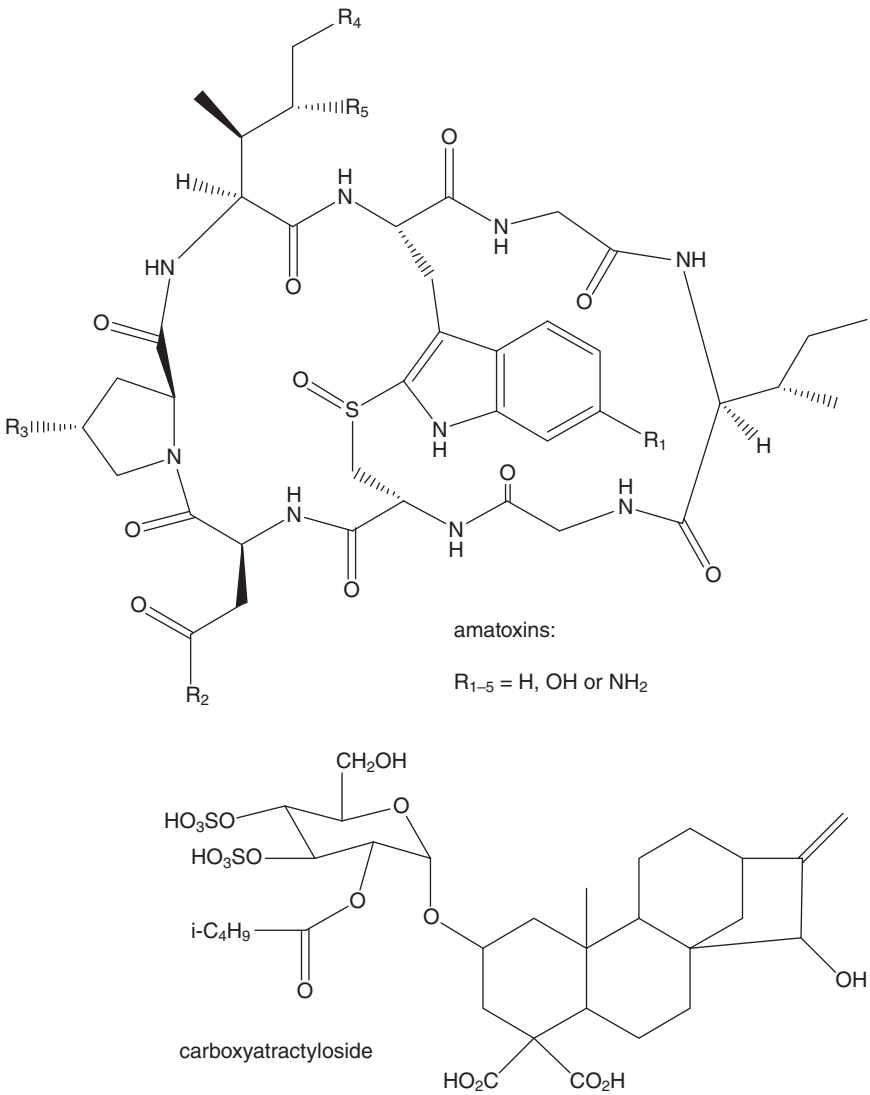


Fig. 14.2 The cyclic peptide amatoxins and the sulfonated glycoside carboxyatractyloside.

produced by mushroom species of the *Amanita* and *Galerina* genera. Though human poisoning is relatively common, animal poisonings are probably under-diagnosed with confirmed poisonings only in dogs (Tegzes and Puschner, 2002). The amatoxins generally cause widespread hepatocellular necrosis with disruption and collapse of hepatocellular cords and sinusoids (Liggett and Weiss, 1989). At lower doses, renal involvement is also described. While mushrooms are not common contaminants of livestock feed, it is conceivable that accidental ingestion may occur, and amatoxin-related poisoning should probably be considered as a differential diagnosis with other causes of similar hepatocellular pathology such as leptospirosis or Theiler's disease.

Hepatocellular response varies from single-cell necrosis or apoptosis to massive necrosis involving hepatocytes and/or the biliary epithelium. The location and types of hepatic cells affected often depend on the toxin and physiologic state of the liver. For example, hepatic necrosis produced by the glycosidic carboxyatractylosides (Fig. 14.2) of *Xanthium cananillesii* (cocklebur), that have poisoned animals and humans, begins primarily as degeneration and necrosis of centrilobular hepatocytes (Seawright *et al*., 1982; Turgut *et al*., 2005). This is contrasted by the degeneration and necrosis of periportal hepatocytes produced by aflatoxin B1 (Fig. 14.1) produced by *Aspergillus flavus* (Bannasch *et al*., 1985).

The clinical presentation of acute liver disease varies from depression to neurologic signs. Jaundice (icterus) is most easily observed in the conjunctiva and non-pigmented mucosal surfaces. Hypoalbuminaemia and hyperammonaemia also develop, leading to peripheral oedema, ascites, coagulopathies, haemorrhage and hepatic encephalopathy. With severely impaired hepatic function, other organ systems fail, resulting in cardiovascular and renal failure and ultimately death. Common post-mortem findings include a swollen, red liver with oedema and ascites.

14.3.2 Chronic toxicity

Chronic hepatocellular injury is generally caused by less potent or lower doses of toxins with longer durations. Since most chronic poisonings are also characterized by hepatocellular degeneration and necrosis, that may have similar lobular patterns to acute hepatocellular necrosis, the primary target continues to be the hepatocyte. However, the necrosis of these chronic lesions is also accompanied, and at times obscured, by atrophy, fibrosis, steatosis, hepatocellular nodular hyperplasia, biliary proliferation or inflammation. The clinical signs of chronic hepatic disease include jaundice, photosensitization, hepatic encephalopathy, and behavioural changes. Since chronic liver disease is progressive, nearly all hepatic toxins and other hepatic diseases produce similar end-state cirrhotic lesions that make an aetiologic diagnosis difficult.

14.3.3 Hepatogeneous photosensitivity

There are two major forms of photosensitization by which the less pigmented areas of the body become highly sensitive and seriously damaged by sunlight. Primary photosensitization involves ingestion of photodynamic agents, or pro-photodynamic agents requiring metabolic activation, that subsequently are circulated to the skin where they interact directly with sunlight, producing dermatitis. Secondary photosensitization, or hepatogeneous photosensitization, involves primary damage to the parenchymal or the biliary system of the liver (Kellerman *et al*., 1994) and the subsequent increased systemic circulation of phylloerythrin, a photodynamic chlorophyll metabolite usually excreted in the bile.

Examples of parenchymal or hepatocyte damage leading to photosensitization include the mycotoxicoses mentioned on pages 360–362, i.e., acute bovine liver disease, blue-green algae (microcystins) and facial eczema (sporidesmin). By contrast, and for example, primary damage to the membranes of the small bile ducts is caused by the lantadenes (Fig. 14.3) derived from *Lantana* spp. (Pass, 1991; Sharma, 1994). These pentacyclic triterpene acids require activation via hepatic metabolism and cause retention of bile (intrahepatic cholestasis). The biliary system-related pathogenesis can also involve biliary occlusion with crystalline deposits derived from steroidal saponins (Fig. 14.3) within the plant, e.g., 'alveld' related to *Narthecium ossifragum* in Norway (Flåøyen *et al*., 1991), 'geeldikkop' related to *Tribulus* spp. in South Africa (Kellerman *et al*., 1991, 1994), *Brachiaria* spp. and *Panicum* spp. in Australia (Wilkins *et al*., 1994), *Panicum virgatum* (switchgrass) in the USA (Stegelmeier *et al*., 2007) and *Brachiaria decumbens* (signal grass) and other species in New Guinea (Low *et al*., 1994) and Brazil (Riet-Correa *et al*., 2007).

14.4 Causative agents of hepatotoxicity

As a consequence of the complexity of the liver and its primary role in metabolism of digested food components, the liver can be assaulted by numerous infectious, immunologic and toxic diseases with varying consequences for the health and well-being of the whole body. This is briefly exemplified by the following plant-associated hepatotoxicities.

14.4.1 Fungal metabolites

There are numerous fungus-derived hepatotoxins that contaminate animal feed or pasture plants. For example, the aflatoxins, e.g. aflatoxin B1 (Fig. 14.1) produced by *Aspergillus flavus* and *A. parasiticus*, have been extensively studied and reviewed. The fumonisins (see Chapter 11), produced by *Fusarium moniliforme*, are a family of polyhydroxylated alkylamines diesterified with tricarballylic acid (Fig. 14.4). These are multiorgan-targeting

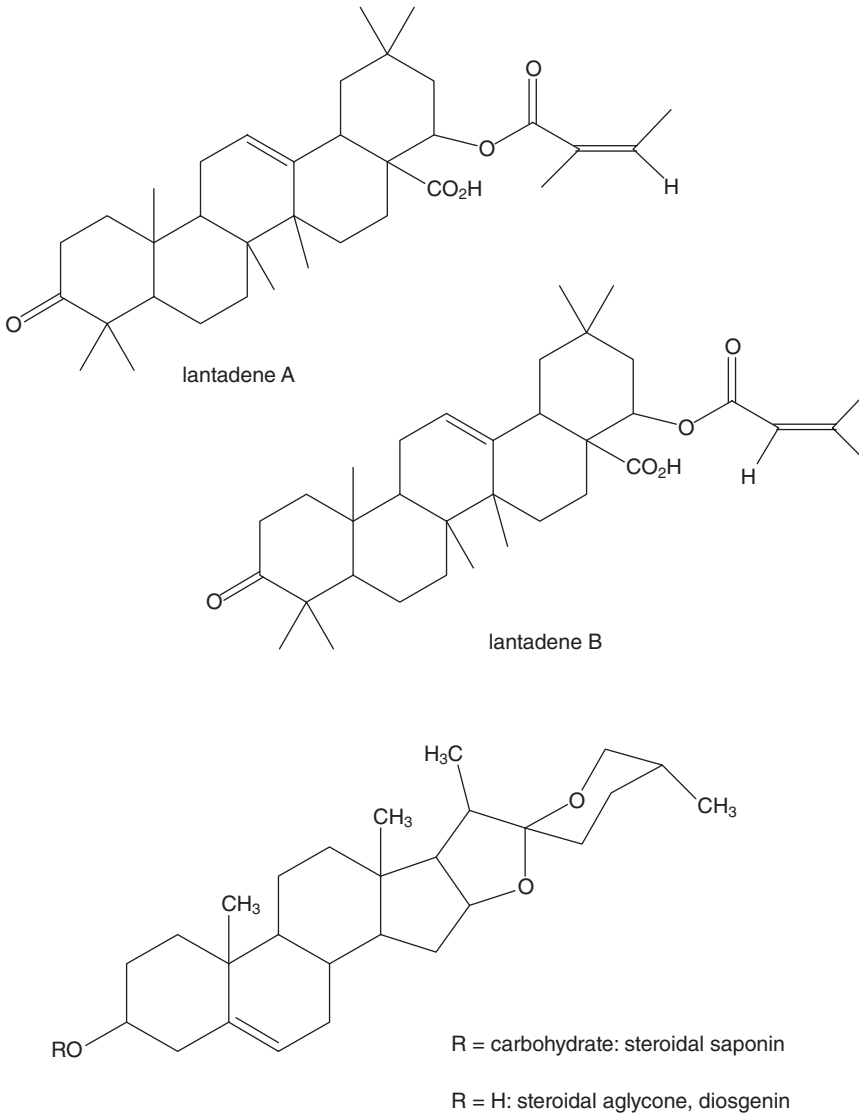


Fig. 14.3 Hepatotoxic steroids: triterpenoid lantadenes from *Lantana* spp. and the sapogenin diosgenin, released by *in vivo* hydrolysis of the corresponding saponin.

toxins, including the liver, that also display species-specific effects (Diaz and Boermans, 1994). For example, acute high doses of fumonisins lead to liver pathology in horses whereas leukoencephalomalacia develops as a result of long-term, low-level exposure (Kellerman *et al.*, 1990; Ross *et al.*, 1993) (for details on mycotoxins see also Chapters 11 and 12).

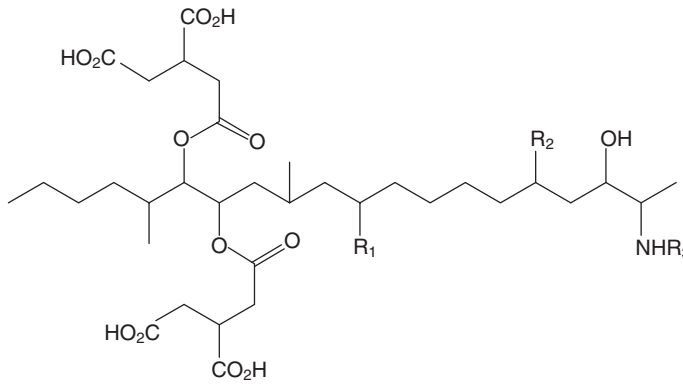


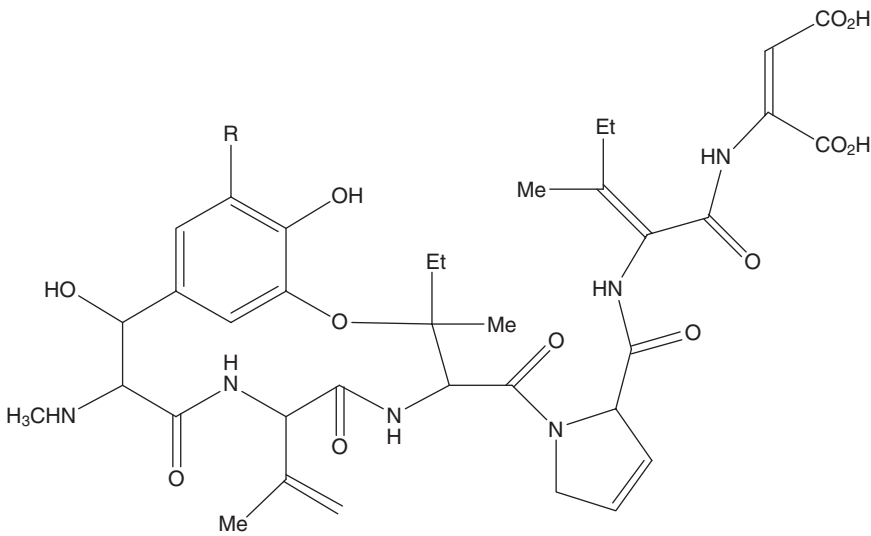
Fig. 14.4 The fumonisins: R_1, R_2 = H or OH, and R_3 = H or CH_3O.

Lupinosis

Lupinus spp.-related teratogenesis due to constituent quinolizidine and piperidine alkaloids has been well documented for the native *Lupinus* spp. in the western USA (Panter, 1993). However, cultivated lupins that have been bred to be low in such alkaloids can become infected with the saprophytic fungus *Diaporthe toxica* (formerly *Phomopsis leptostromiformis*). Ingestion of the fungal metabolites, the hexapeptide phomopsins (Fig. 14.5), can lead to a liver disease in sheep called lupinosis (Allen *et al*., 1985; Cockrum *et al*., 1994; Edgar, 1991). The phomopsins inhibit the cellular process of polymerization of α and β tubulin dimers to form microtubules. Since microtubules are essential components of the mitotic spindle involved in chromosome division within eukaryotic cells, cell mitosis is blocked in metaphase. This is similar to the activity of colchicine isolated from *Colchicum* spp. (Kiselev and Yavich, 1990).

Intoxication of sheep usually occurs once the lupine seed has been harvested and the sheep are allowed to graze the lupine stubble. Cattle are also affected by the phomopsins, leading to two main syndromes (Allen, 1981). The more commonly observed syndrome is a fatty liver and ketosis that occurs in pregnant, nutritionally challenged cows. The second syndrome, involving liver cirrhosis, more closely resembles the liver disease lupinosis in sheep but with a higher incidence of observed photosensitization. Because of a similarity in lesions, and detection of the fungus, an involvement of the phomopsins has been tentatively suggested in the aetiology of hepatic fatty cirrhosis, with associated hepatic encephalopathy, in cattle, sheep and native ruminants such as the pronghorn antelopes in the western USA (Helman *et al*., 1993).

Since lupine seeds, for livestock and human consumption, can also be infected with the fungus and hence contaminated with the phomopsins (which are resistant to processing, including cooking), regulations have



R = Cl: phomopsin A

R = H: phomopsin B

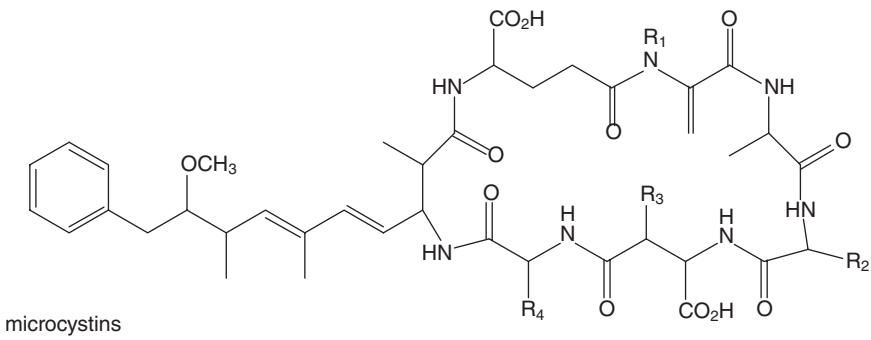
Fig. 14.5 Structures of the phomopsins, the causative agents of lupinosis in sheep.

been established to protect consumers. In Australia, the maximum acceptable level of phomopsins in lupines is 5 parts per billion (ppb) (FSANZ, 1999, 2001a). The regulation also applies to other foods that can be infected by the fungus such as chestnuts and mangoes.

Acute bovine liver disease

Some of the initial differential causes of a fatal hepatopathy of dairy cattle that occurs across southern Australia (Gunn and Clarke, 2003) included microcystins, sporidesmin and an association with the grass *Cynosurus echinatus* (rough dog's tail). The microcystins (Fig. 14.6) are hepatotoxic, cyclic peptides produced by cyanobacteria (blue-green algae) such as *Microcystis aeruginosa* (Jackson *et al*., 1985) whereas sporidesmin (Fig. 14.6), a complex dithiopiperazinediketone and the causative agent of hepatogenic facial eczema, is produced by the saprophytic fungus *Pithomyces chartarum* in pastures (Russell, 1960; Smith and Embling, 1991).

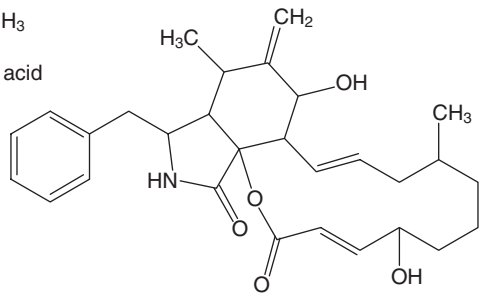
Early and ongoing signs of the intoxication included a drastic reduction in milk production. Affected animals showed effects of photosensitization and some neurological abnormalities such as abnormal tongue protrusion and licking behaviour (*Animal Health Surveillance Quarterly*, 2006; S. M. Colegate, personal observation). Acutely affected animals are, however,



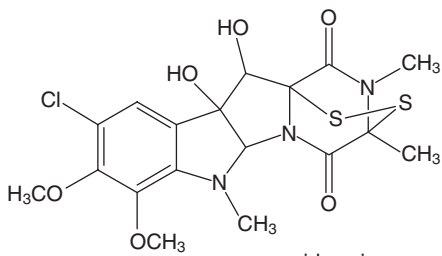
microcystins

R_1 and R_3 = H or CH_3

R_2 and R_4 = amino acid



cytochalasin B



sporidesmin

Fig. 14.6 Structures of the microcystins, sporidesmin and the cytochalasins, considered in the aetiology of acute bovine liver disease.

found dead prior to any onset of photosensitization. Mostly reported in dairy cattle, the intoxication has been observed in beef cattle, but has not been confirmed in sheep. This apparent difference in species susceptibility may be due to grazing habits rather than an actual difference in response to the hepatotoxins.

Careful examination of the pathology contra-indicates blue-green algae (microcystins) or a facial eczema-like (sporidesmin) involvement. An *in vitro* bioassay using rat hepatocytes in OptiCells™ was used to investigate extracts of *Cynosurus echinatus* (rough dog's tail) and a fungus, *Drechslera biseptata*, cultured from *C. echinatus* collected at the time and site of an

intoxication (Aslani *et al*., 2006). The subsequent health of the hepatocytes, following exposure to extracts, was monitored in 'real time' by microscopic examination of the cells within the OptiCell™. Hepatocytotoxic effects were correlated with putative cytochalasin-like compounds (Fig. 14.6) isolated from the methanolic extracts of the plant and the fungus (Aslani *et al*., 2006). Further work is required to confirm the structures of the hepatocytotoxic compounds and to demonstrate induction of acute bovine liver disease with the isolated compounds.

14.4.2 Tannins

While tannins have been usually associated with anti-nutritional effects (Blytt *et al*., 1988; Mansoori and Acamovic, 1998) some are hepatotoxic. For example, the α and β isomers of punicaligan (Fig. 14.7) were isolated from *Terminalia oblongata* (yellow-wood) using the induction of liver lesions (severe periacinar necrosis) in mice as a bioassay to guide the extraction and purification (Oelrichs *et al*., 1994). Notably, a related tannin, terminalin (Fig. 14.7), isolated from the same plant induced adverse renal effects rather than liver damage. The combined effect of the isolated tannins in inducing liver and kidney damage was consistent with the field observations of intoxication that involved both a hepatic and a renal syndrome (Filippich *et al*., 1991). Other plants that produce hepatotoxic tannins include the economically significant *Thiloa glaucocarpa* in Brazil (Itakura *et al*., 1987; Riet-Correa *et al*., 2007).

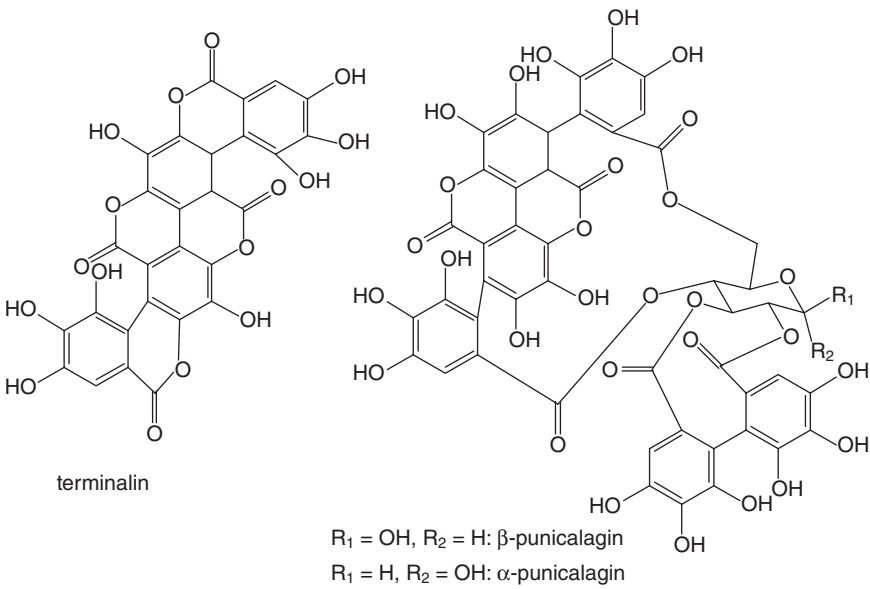


Fig. 14.7 Hepatotoxic tannins from *Terminalia oblongata*.

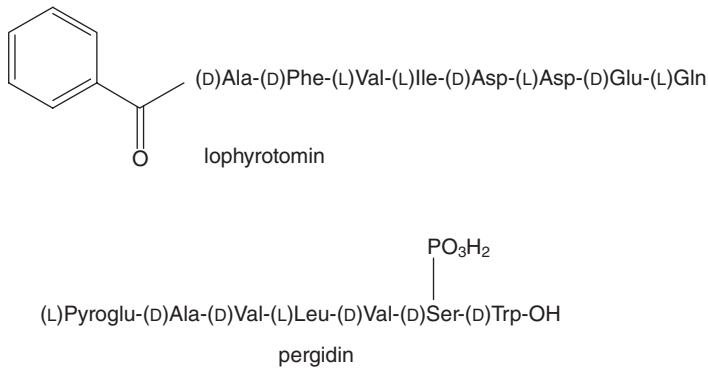


Fig. 14.8 Hepatotoxic peptides from sawfly larvae.

14.4.3 Sawfly peptides

Hepatotoxic reaction associated with the ingestion of sawfly larvae by cattle, sheep, pigs, dogs and laboratory animals has been reported in Australia, South America and Denmark (Oelrichs *et al*., 1999). Lophyrotomin and pergidin are linear peptides that contain both L- and D-amino acids (Fig. 14.8). Lophyrotomin is an octapeptide isolated as the main toxin from dried larvae of *Lophyrotoma interrupta* (iron-bark tree sawfly) from Australia and from the birch tree sawfly (*Arge pullata*) from Denmark. Pergidin is a phosphorylated heptapeptide isolated as the main toxin from dried larvae of *Perreyia flavipes* (mata porco sawfly) from South America, particularly Uruguay, Brazil, Argentina, Paraguay and Venezuela.

The liver pathologies induced by lophyrotomin or pergidin are different. The former results in a periportal haemorrhagic necrosis, while pergidin causes a periacinar coagulative necrosis (Oelrichs *et al*., 2001). It has been suggested (Oelrichs *et al*., 1999, 2001) that the stability of these hepatotoxins to heat and enzymatic degradation, due to their unusual configuration and their strong chemical binding properties, may lead to residues in animal tissues that presumably might enter the human food supply. It has further been suggested (Oelrichs *et al*., 2001) that this latter potential needs to be a consideration when evaluating the environmental release of these sawflies for the control of *Melaleuka quenquinervia* that has become a weed pest in the south-eastern USA.

14.5 The hepatotoxic pyrrolizidine alkaloids

In general, pyrrolizidine alkaloids (PAs) have a central core of two, fused, fully saturated five-member rings with a nitrogen atom at one of the ring junctions (Fig. 14.9). Examples range from compounds in which the PA core

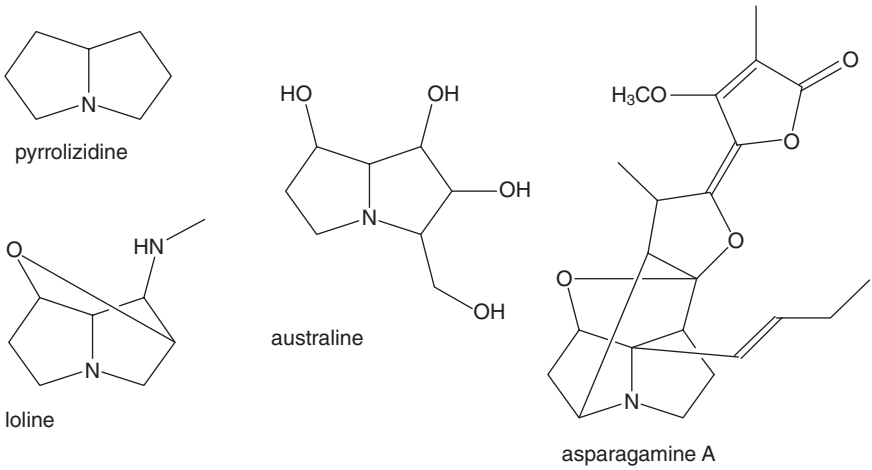


Fig. 14.9 Examples of pyrrolizidine alkaloids.

is the main structural element, such as in the polyhydroxylated PA glycomimic australine (Fig. 14.9) and in the staggers-inducing amino PA loline (Fig. 14.9), to compounds in which the PA ring system is embedded within a complex multi-ring system such as in the insecticidal asparagamine A (Fig. 14.9). However, the focus of this chapter is the hepatotoxic dehydropyrrolizidine alkaloids (DHPAs) and their *N*-oxides. These are monoesters, open-chain diesters or macrocyclic diesters of 1-hydroxymethyl-7-hydroxy-1,2-dehydropyrrolizidine (retronecine and heliotridine), or cyclic diesters of its unbridged macrocyclic analogue (otonecine) (Fig. 14.10). The 1,2 unsaturation and the hydroxylation or esterification at C7 and C9 are essential structural requirements for pyrrolizidine alkaloid-related hepatotoxicity.

14.5.1 Sources of hepatotoxic pyrrolizidine alkaloids

Hepatotoxic PAs have been mainly associated with genera and species of the Boraginaceae, Asteraceae (Compositae), Leguminosae (Fabaceae) and Apocyanaceae (IPCS, 1988; Bull *et al*., 1968; Rizk, 1991). Some of the more relevant genera include *Senecio*, *Heliotropium*, *Echium*, *Symphytum*, *Crotalaria* and *Trichodesma*. Species of these plant genera can be present in natural grazing situations in which their contribution to the ecosystem has been regulated by evolutionary pressures. However, while these naturally occurring plants still present problems for livestock agriculture, e.g. *Senecio* spp. on open rangeland in the western USA, it is those plants that have been transferred from their evolutionary niche that have the propensity to become noxious weeds that take over large tracts of land, displacing native species, changing the ecosystem and posing economic problems for

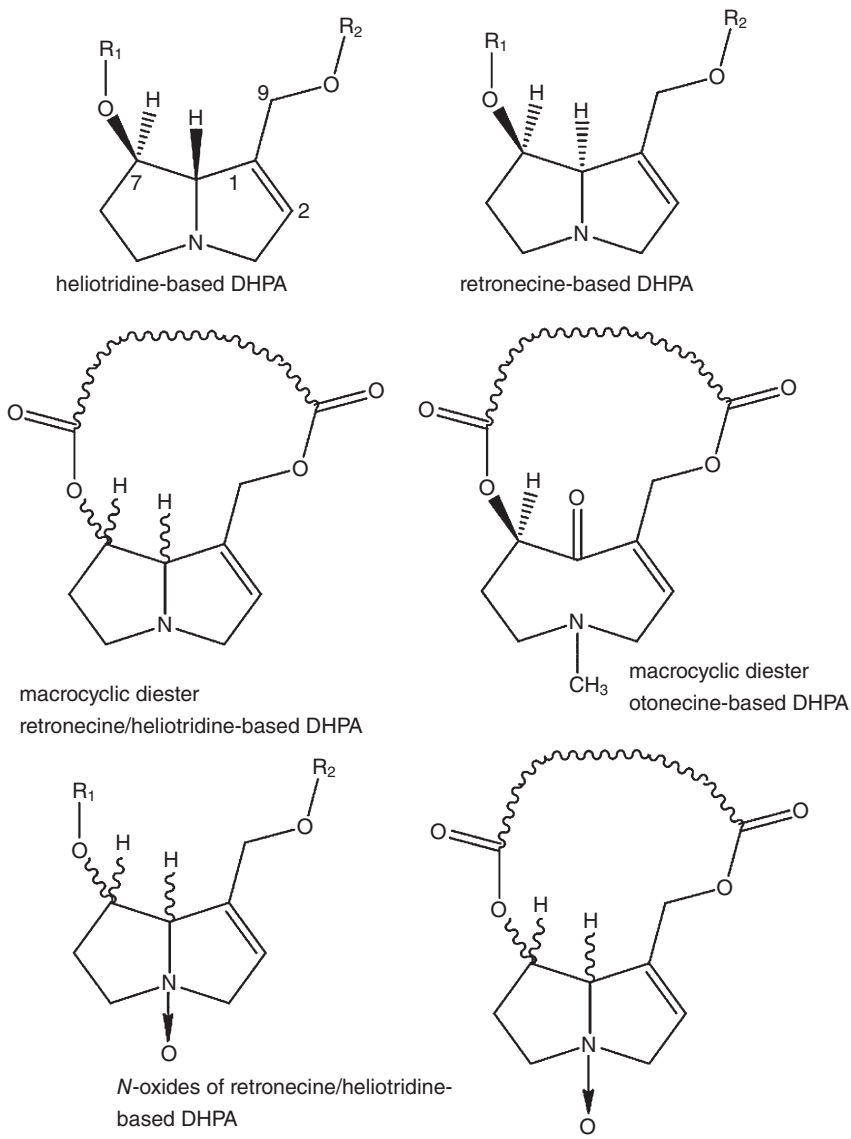


Fig. 14.10 Basic core structures of the hepatotoxic dehydropyrrolizidine alkaloids and their *N*-oxides.

cropping and livestock agriculture. Such plants include *Echium plantagineum* (Paterson's curse; Salvation Jane), *E. vulgare* (alpine blue borage), *Heliotropium europaeum* (heliotrope; potato weed), *Senecio madagascariensis* (fireweed), *S. jacobaea* (ragwort), *Ageratum* spp., and *Chromalaena* spp.

14.5.2 Chemistry and basic toxicological aspects

There are in excess of 350 described structures for the DHPAs that vary in the composition of the esterifying acids and the configuration of substituents on those acids (Bull *et al*., 1968; Smith and Culvenor, 1981; Rizk, 1991). Combined with the relative configuration of the C7 and C8 hydrogens, this structural flexibility produces many isomeric DHPAs such as echinatine/rinderine/lycopsamine/intermedine, and isobaric DHPAs (triangularine/senecionine/doronenine) (Fig. 14.11).

The DHPAs also occur, usually as the dominant form in plants, as their *N*-oxides. While *N*-oxidation of DHPAs is one of the hepatic detoxification

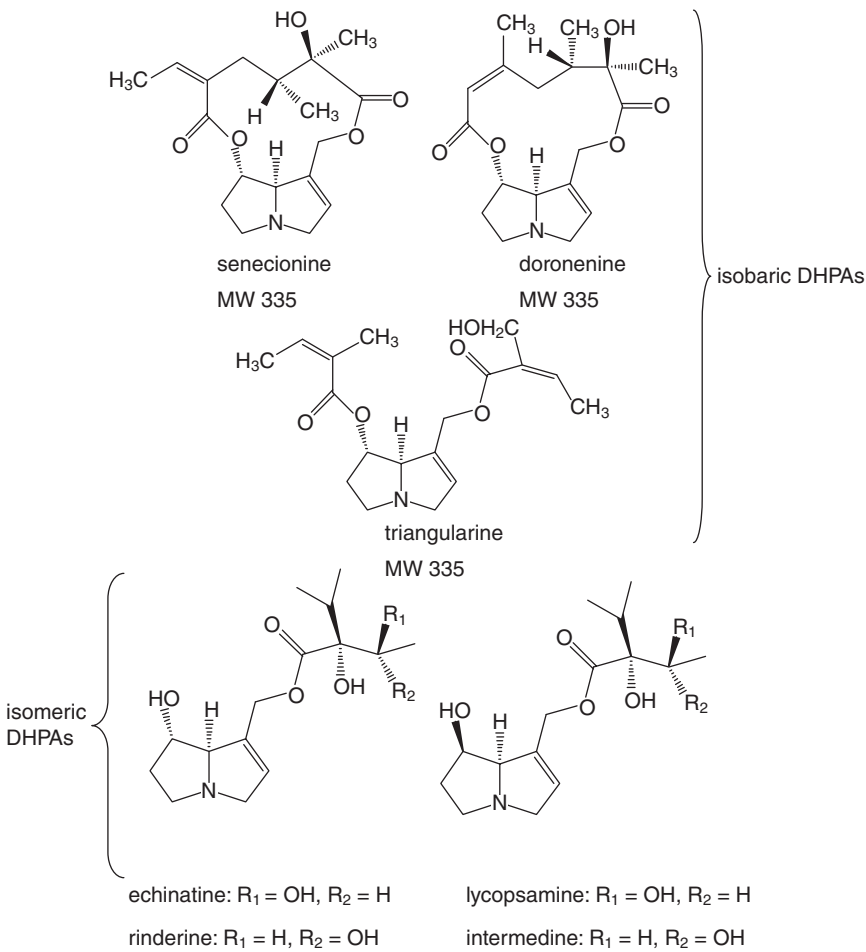


Fig. 14.11 Examples of isobaric dehydropyrrolizidine alkaloids, i.e., senecionine, doronenine and triangularine all with a molecular weight of 335 Da, and isomeric dehydropyrrolizidine alkaloids, i.e., the heliotridine-based echinatine and rinderine, and the retronecine-based lycopsamine and intermedine.

pathways resulting in an increased rate of elimination of the DHPAs, it has been shown that dietary exposure to the naturally occurring DHPA *N*-oxides still results in hepatotoxicity (Chou *et al*., 2003; Mattocks, 1972, 1986; Wang *et al*., 2005). This is presumably due to *in vivo* reduction of the *N*-oxide to the parent DHPA before exposure to the liver (Mattocks, 1972). Therefore, any analytical and dose–response assessment of the potential of ingested plants to induce toxic effects in livestock or in humans needs to take into account the *N*-oxides (Cao *et al*., 2008).

Extraction and analysis

Due to the basic nature of the nitrogen and the water-solubility of their *N*-oxides, the alkaloids are extracted from matrices or partitioned from crude organic solvent extracts using dilute aqueous acid solutions. Basification of the aqueous extract and re-extraction with an immiscible organic solvent such as chloroform then allows recovery, at a higher purification, of the DHPAs. However, this will not allow recovery of the water-soluble *N*-oxides that must be analytically captured if a more accurate, meaningful assessment of toxicity is to be developed (Cao *et al*., 2008).

The traditional approach to determining the level of *N*-oxide relative to the free parent bases is to process two aliquots of the aqueous acid solubles from the plant (or its crude organic solvent extract). One aliquot is simply basified and extracted with an immiscible solvent to capture the natural free base component of the extract. The other acidified aliquot is treated with zinc dust to reduce all the *N*-oxides to their parent free bases prior to basification and extraction. The quantitative difference in DHPA content between the two aliquots represents the relative contribution of the *N*-oxides to the overall DHPA content. However, more recently, solid phase extraction (SPE) of the DHPAs and their *N*-oxides followed by LCMS analysis have been applied to more efficiently determine the DHPA and DHPA–*N*-oxide content of plant material (Colegate *et al*., 2005). To confirm the suspected presence of *N*-oxides the methanolic sample extract can be simply treated with a redox resin to reduce all *N*-oxides to the free bases prior to re-analysis of the sample. For example, LCMS analysis of a methanolic extract of root material from *Symphytum officinale* (common comfrey) readily reveals the major presence of the *N*-oxides of lycopsamine and one of its diastereoisomers, intermedine (peaks 1), the C7-OH acetylated derivatives of lycopsamine and intermedine (peak 2) and the symlandine and symphytine configurational isomers (peaks 3) (Fig. 14.12). The presence of the respective parent free bases, to about 10% of the amount of the *N*-oxides, is revealed by generating reconstructed ion chromatograms (RICs) from the total ion data. For example, low levels of naturally occurring (as opposed to the deliberately reduced extract) acetyl derivatives (peak 4) are shown (Fig. 14.12 A3). Also shown (Fig. 14.12 B1) is the reduced extract that confirms the *N*-oxide character of the peaks in Fig. 14.12 A1.

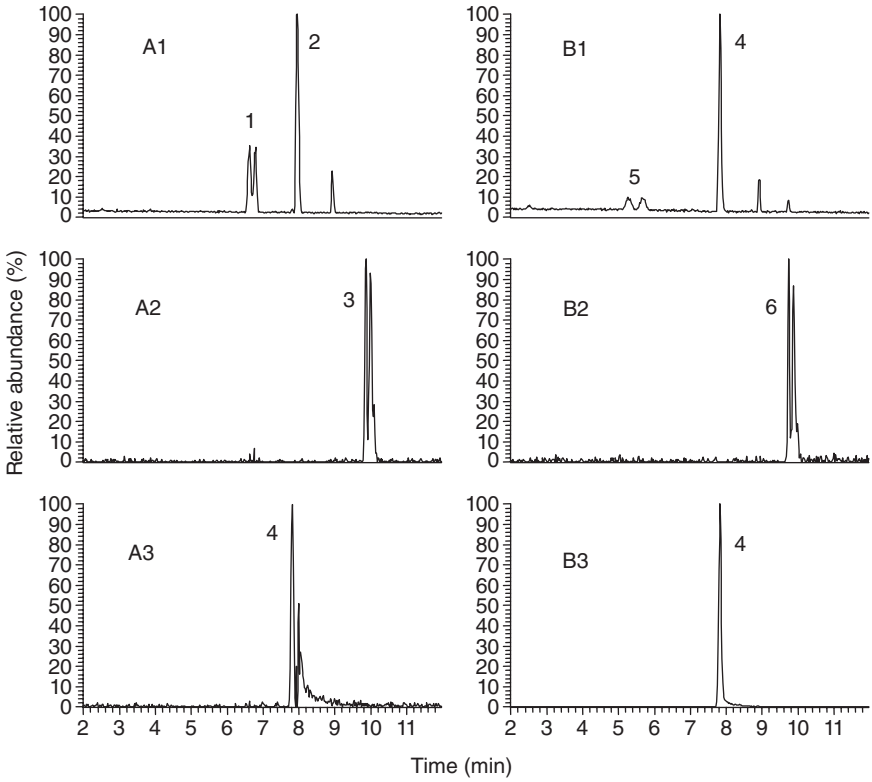


Fig. 14.12 HPLC-esiMS base ion (*m/z* 80–1000) chromatograms of a crude methanol extract of *Symphytum officinale* (comfrey root) (**A1**) and its reduced product (**B1**). Reconstructed ion chromatograms are also shown displaying ions with *m/z* 398 (**A2**), *m/z* 382 (**B2**) and *m/z* 342 (**A3** and **B3**). Peaks 1, 2 and 3 are the *N*-oxides of lycopsamine/intermedine (peaks 5), acetyllycopsamine/acetylintermedine (peak 4) and symlandine/symphytine (peaks 6) respectively. Thus **A2** reveals the presence of low levels of the *N*-oxides of symlandine/symphytine while **A3** reveals a low, natural level of the acetylated lycopsamine and intermedine free bases.

The SPE capture and subsequent LCMS analysis of DHPAs and their *N*-oxides has been applied to the examination of the following:

- Honeys and pollen (Beales *et al*., 2004, 2007; Betteridge *et al*., 2005; Boppré *et al*., 2005, 2008). The example (Fig. 14.13) using honey and pollen derived from *Echium vulgare* clearly shows the significant presence (labelled peaks in Fig. 14.13) of open-chain diester DHPA free base and *N*-oxides in the honeys (Fig. 14.13(a)) that are also abundantly present in the pollen extract (Fig. 14.13(b)).
- DHPA-producing pasture plants (Boppré *et al*., 2008; Colegate *et al*., 2005; Gardner *et al*., 2006). The example (Fig. 14.14) demonstrates the difference in DHPA profile between samples of *Senecio madagascarien-*

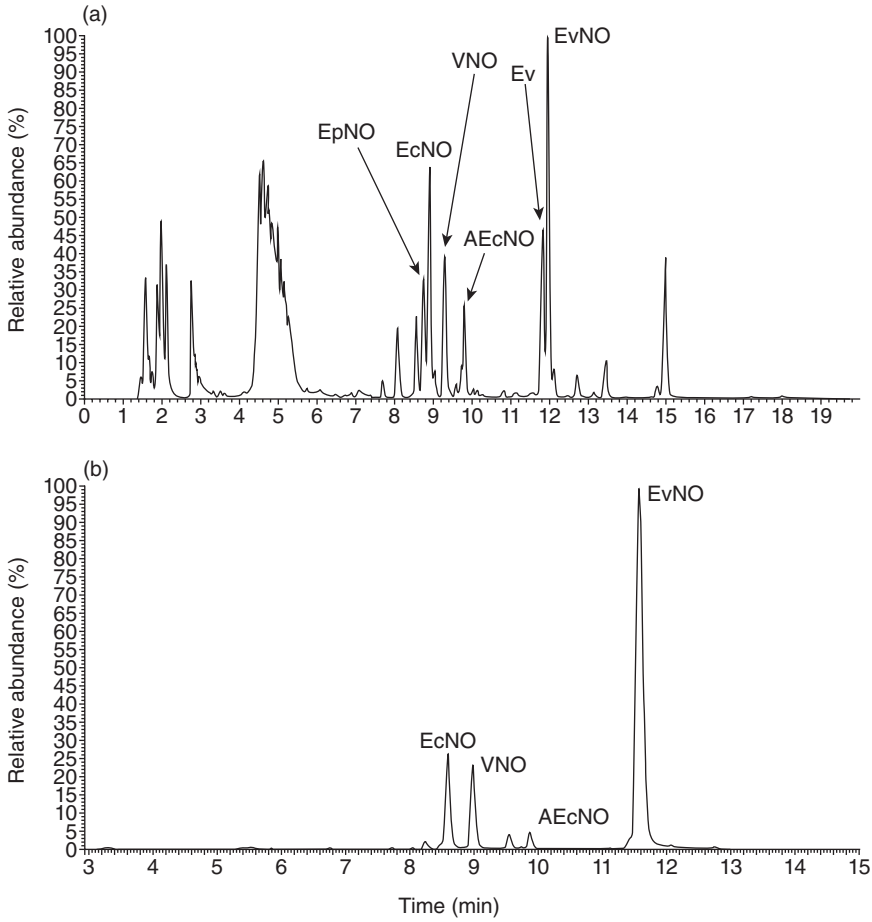


Fig. 14.13 HPLC-esiMS base ion (*m/z* 200–500) chromatograms of SPE-processed honey (a) and pollen (b) derived from *Echium vulgare*. The labelled peaks in the honey are the free base and *N*-oxides of open-chain diester DHPAs that are also clearly and abundantly present in the pollen. Ep = echimiplatine; Ec = echimidine; V = vulgarine; AEc = acetylechimidine; Ev = echivulgarine; suffixed by NO = *N*-oxide.

sis (fireweed) collected from pastures in different areas of New South Wales, Australia, in different years.

- Hay samples contaminated with a *Senecio* sp. and suspected of poisoning livestock. The potential for poisoning livestock was confirmed by the observation of significant levels (*ca*. 300 ppm) of DHPA *N*-oxides (*m/z* 352 and 398) in addition to higher levels of a 1,2-saturated pyrrolizidine alkaloid (*m/z* 354) tentatively identified as sarracine-*N*-oxide (Fig. 14.15, Colegate, Gardner and Knight, unpublished).

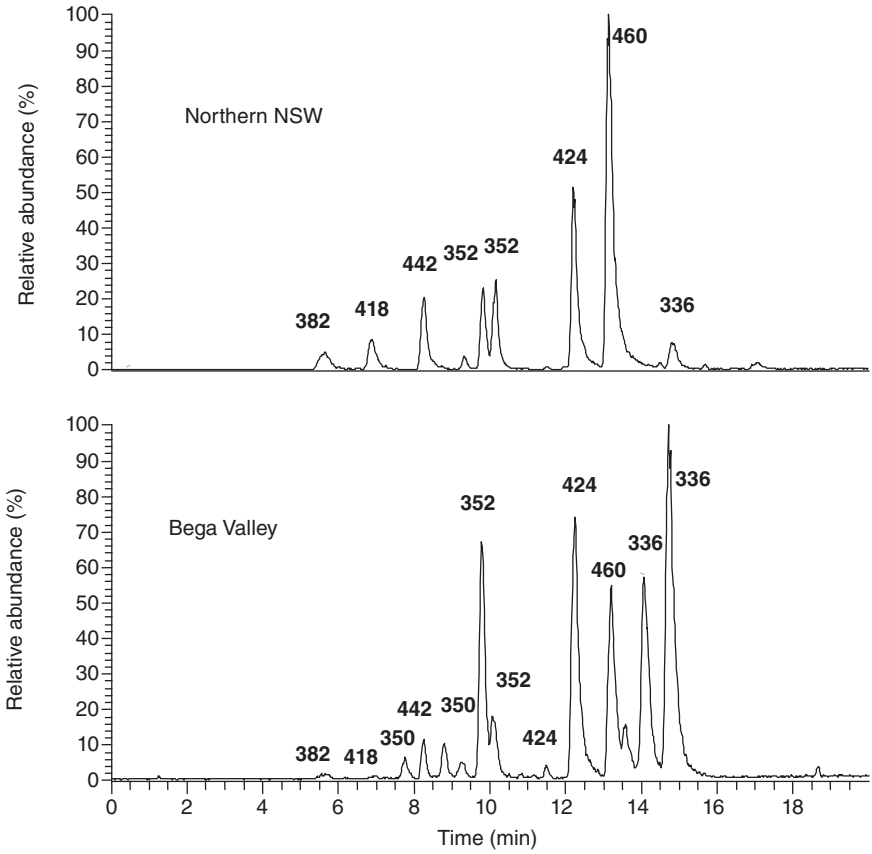


Fig. 14.14 An HPLC-MS comparison of a zinc/acid-reduced alkaloidal extract of fireweed (*Senecio madagascariensis*) collected from pastures in the Bega Valley, New South Wales, Australia in about October 2006 with that collected from pastures in northern New South Wales, Australia (Gardner *et al*., 2006). The **bold numbers** are the MH^+ values for each peak and correlate to macrocyclic diester DHPAs (Colegate and Gardner, unpublished).

In contrast to the qualitative assessments of samples for the presence of DHPAs, a major consideration when quantitatively analysing samples is the commercial lack of pure authenticated standards. This is compounded by the sheer diversity of DHPAs that are produced by the various plant sources and, in many cases, by instability of the pure compounds. This has been managed in some cases by describing levels of DHPA as equivalents of a known, stable, authenticated DHPA standard, preferably one that is similar in structure including *N*-oxide character (Boppré *et al*., 2005; Betteridge *et al*., 2005). While this approach provides a good comparison of relative

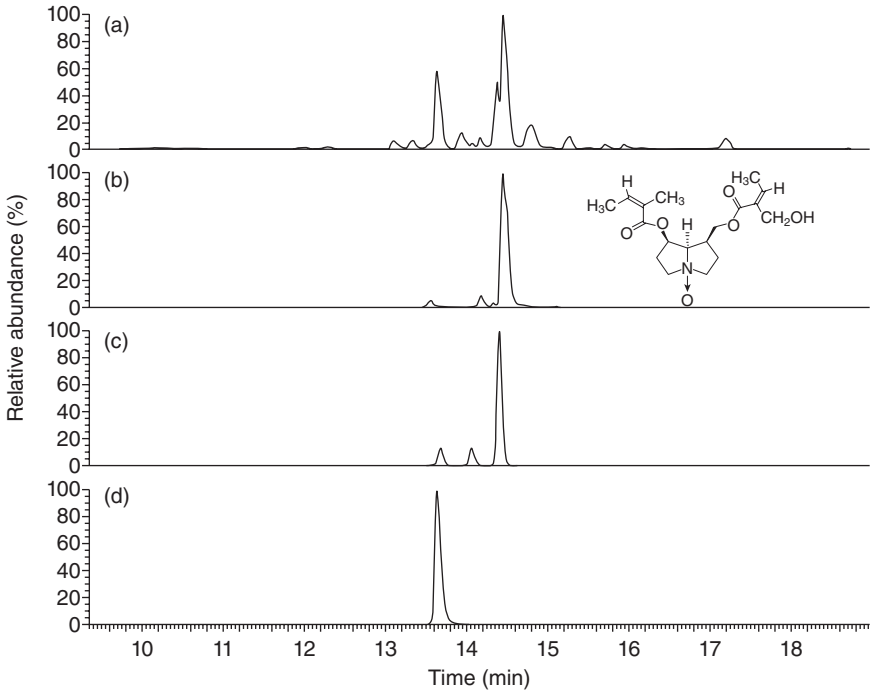


Fig. 14.15 (a) HPLC-esiMS base ion (*m/z* 80–1000) chromatogram of a methanolic extract of suspected toxic hay. Also shown are reconstructed ion chromatograms displaying (b) *m/z* 354, probably the non-toxic, saturated pyrrolizidine alkaloid sarracine; and the potentially toxic dehydropyrrolizidine alkaloids with *m/z* 352 (c) and *m/z* 398 (d).

amounts of specific DHPAs between samples, it does not, even with careful selection of quantitative standards for the DHPAs and their *N*-oxides, necessarily provide an accurate absolute level of the specific DHPAs, since the response factors can be quite different (Boppré *et al*., 2005). To obviate the need for individual standards, approaches have been developed to quantitate a common entity derived from the DHPAs. For example, Kempf *et al*. (2008) described the strong cation exchange SPE of DHPAs and their *N*-oxides followed by two reduction steps (zinc/acid to reduce *N*-oxides and lithium aluminium hydride to reductively cleave off the esterifying necic acids) and silylation of the generated heliotridine or retronecine.

A comprehensive review of analytical methods for pyrrolizidine alkaloids has recently been prepared by Crews *et al*. (2010) and the different analytical procedures are also addressed in Chapter 12.

Basic toxicology

The DHPAs are protoxins, requiring bioactivation to the toxic entity. Once absorbed, hepatic CYP450 enzymes reduce the DHPAs to the 'pyrrolic',

didehydropyrrolizidine derivatives. These electrophilic 'pyrroles' react with macromolecules (e.g. proteins and nucleic acids) in the hepatocytes and in the endothelial cells of the adjacent sinusoids and small hepatic veins. This leads to hepatocellular and biliary injury, including the characteristic necrosis, fibrosis, biliary duct epithelial cell proliferation, megalocytosis, veno-occlusive disease and cirrhosis. Some DHPAs have also been shown to be pneumotoxic, carcinogenic and genotoxic (for review see Fu *et al*., 2004).

As a consequence of the need to be metabolically activated, and the differences in the stability and relative reactivity of the resulting 'pyrrolic' didehydroalkaloids, not all DHPAs are equally toxic. Additionally, not all animal species are equally susceptible, since species can activate, detoxify and eliminate the DHPAs at different rates. For example, while cattle and horses are quite susceptible, needing, for example, a dry-mass intake of *Senecio jacobaea* of only about 5% of their body weight to be lethal, sheep and goats are more resistant, requiring intakes of several hundred percent of bodyweight (Mattocks, 1986; Cheeke, 1988).

The clinical and pathological manifestations of specific DHPA intoxication also differ between species. These can range from effects, and consequences of such effects, on the liver, lungs and kidneys. There are also a host of secondary effects related to exposure to the DHPAs (Hooper, 1978). Significantly, in a review of pyrrolizidine alkaloids in the human diet (Prakash *et al*., 1999) it was reported that human hepatocytes respond somewhat differently to the DHPAs in that they do not show the antimitotic effect that leads to the megalocytosis characteristic of DHPA intoxication of animals. Furthermore, in the review, Prakash *et al*. (1999) conclude that human hepatocytes may be relatively resistant to the genotoxic effects of metabolized DHPAs and that DHPAs are not carcinogenic to humans.

14.5.3 Hepatotoxic pyrrolizidine alkaloids in livestock feed

The exposure of livestock to DHPAs and the effects therefrom have been discussed by Stegelmeier *et al*. (1999, 2009). Since most DHPA-producing plants are not highly palatable to most livestock, poisoning more often occurs when the plants or plant parts are included in prepared forages or when their seeds contaminate grain. Less frequently, animals are poisoned when they are forced to eat DHPA-producing plants when no other forages are available.

Susceptibility of livestock to poisoning

Different livestock species have vastly different susceptibilities to poisoning by DHPAs. The susceptibility is influenced by species-specific metabolism (including the ability of the liver to synthesize the 'pyrrole' metabolites, and the rate of proliferation, growth and metabolism of hepatocytes), age, sex, and other temporary factors such as biochemical, physiologic and nutritional status.

Young animals are generally more susceptible to DHPAs than aged adults. Neonatal and nursing animals have been reported to develop fatal hepatic disease while the lactating dams were unaffected (Small *et al*., 1993). From a nutritional status perspective, animals that have marginal nutrition are more likely to develop disease. In addition, sheep are particularly prone to a synergistic effect of dietary DHPAs and copper leading to excess storage of copper and a fatal haemolytic crisis (Howell *et al*., 1991).

From a species perspective, some reported toxicity indices are pigs 1, chickens 5, cattle and horses 14, rats 50, mice 150, and sheep and goats 200 (Hooper, 1978). In another study, the toxic dose of *Senecio* sp. was 20 times higher for sheep and goats than the dose that killed cattle (Dollahite, 1972). Due to the relative resistance to DHPA intoxication by sheep and goats, they can be used with care, since there can be toxic sequelae (Harris, 1998), as biocontrol agents to graze pastures that could poison horses and cattle (Dollahite, 1972; Sharrow and Mosher, 1982).

Plant variation and plant–animal interactions

Usually plants are most toxic in the early bud stage when beginning to flower. Nonetheless, there are large variations in DHPA concentrations between years and locations (Johnson *et al*., 1985) that make it intrinsically difficult to predict when plants will contain potentially toxic levels of DHPAs.

Toxicity is directly related to ingestion of the plant. The amount and rate at which animals eat plants is related to palatability, including any addictive consumption, and the availability of alternative and preferred feed. For any specific plant species, both palatability and the availability of other feed can vary with the season, location and weather. Different populations of the same species can therefore differ greatly in their apparent (if not actual) ability to cause poisoning. For example, horses, belly-deep in *Echium plantagineum* in Western Australia, seemed unaffected, whereas several horses died from *Echium plantagineum*-related liver failure following regrowth after bushfires in the Australian Capital Territory in 2003 (S. M. Colegate, personal observation; Australian Broadcasting Corporation, 2004).

14.5.4 Clinical signs and pathology of DHPA intoxication

Dose-dependent swelling of hepatocytes is the first cellular indication of DHPA intoxication. With continuing damage, cellular degeneration continues with ultimate loss of cellular homeostasis and necrosis (cell death). Acute intoxication often produces pan-lobular hepatocellular necrosis accompanied by haemorrhage and minimal inflammation (Fig. 14.16). These animals show signs of acute liver failure including anorexia, depression, icterus, visceral oedema and ascites. Serum biochemical changes include massive elevations in AST, SDH, ALK and GGT activities with increased amounts of bilirubin and bile acids.

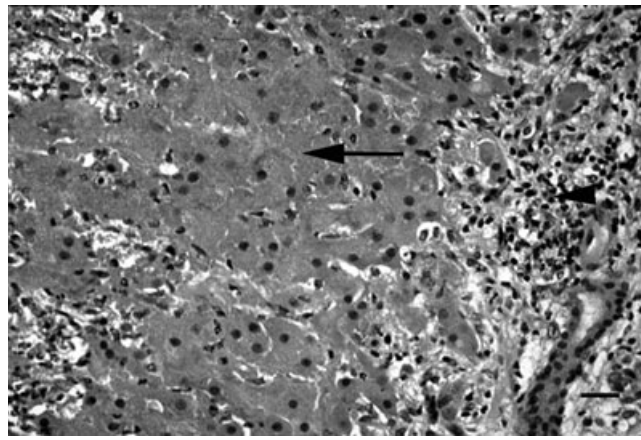


Fig. 14.16 Photomicrograph of the liver of a horse acutely poisoned with houndstongue (*Cynoglossum officinale*). Note the extensive haemorrhagic necrosis (arrow) and collapse of hepatic sinusoids. There is periportal inflammation (arrowhead) with mild proliferation of biliary epithelium and fibroblasts. Bar = 50 microns, H&E stain.

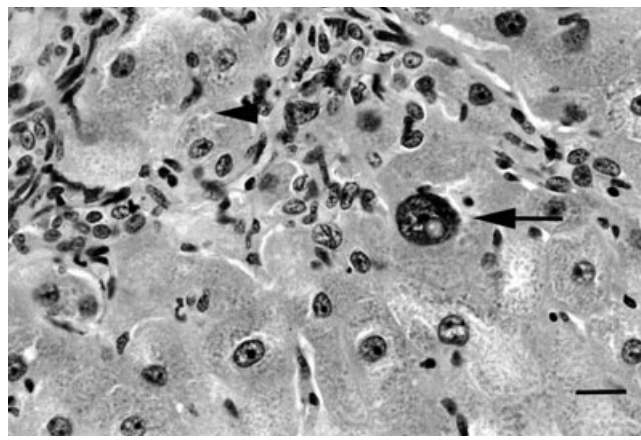


Fig. 14.17 Photomicrograph of the liver of a horse chronically poisoned with houndstongue (*Cynoglossum officinale*). Note the large megalocyte (arrow). Also notice the degenerative hepatocytes that are partially entrapped with proliferative biliary epithelium and fibrosis (arrowhead). Bar = 30 microns, H&E stain.

Chronic poisoning, caused by lower DHPA doses of longer duration, may not be immediately apparent clinically, since animals develop only transient elevations in serum enzyme activities (AST, SDH, ALK and GGT). They may also have only mild elevations in serum bilirubin and bile acids. Hepatic biopsies often have focal hepatocyte necrosis (piecemeal necrosis), minimal peribiliary fibrosis and mild bile duct proliferation. With time, damaged hepatocytes often develop into large megalocytes (Fig. 14.17). Animals may

show no clinical signs and serum biochemistries may be normal for several months or even years after ingestion of the DHPAs. However, due to *in vivo* recycling of the toxic metabolites (see Edgar *et al*., 2011 for a review), hepatocellular damage may continue, resulting in continued hepatocyte necrosis with subsequent inflammation, fibrosis and ultimately cirrhosis. With loss of hepatic function, poisoned animals often do poorly. When such 'hepatic cripples' are subjected to normal stresses, such as pregnancy or lactation, they develop clinical liver failure. Clinical signs such as photosensitivity, icterus, or increased susceptibility to other hepatic diseases such as lipidosis or ketosis are common.

The progressive nature of chronic DHPA intoxication suggests that long-term, low-level exposure to DHPAs has cumulative effects. However, little is known about what doses or durations will be damaging, or the eventual outcomes of subclinical intoxication on growth or productivity.

14.5.5 Diagnosis

Since clinical signs of DHPA-related poisoning are often delayed, they can be difficult to definitively associate with exposure to a specific DHPA-producing plant. Many diagnoses are made using histologic changes alone evaluating hepatic necrosis, fibrosis, biliary proliferation and megalocytosis in liver biopsy samples or slaughtered animals. Unfortunately, these are non-specific changes and a definitive diagnosis of DHPA intoxication is difficult. Therefore, the widespread nature of DHPA-producing plants suggests that DHPA intoxication is under-diagnosed.

Chemical methods using both spectrophotometry and gas chromatography/mass spectrometry have been developed to extract and detect tissue-bound 'pyrroles' (the DHPA metabolites) (Mattocks and Jukes, 1990; Schoch *et al*., 2000). Thus, most acutely poisoned animals will have high concentrations of tissue-bound 'pyrroles', whereas chronically poisoned animals generally have low concentrations that may not be detected. Although chemical detection of 'pyrrolic' adducts has proven to be useful in identifying exposed animals, it lacks sensitivity and is not quantitative.

14.5.6 Treatment or management

Although various treatments and diet supplements have been suggested, none have proven to be effective in livestock. Since clinically overt DHPA-intoxicated animals rarely recover, prevention is the best control measure. Since most poisonings are attributed to contamination of forages or feed, careful inspection of feed is recommended. Contaminated feeds should be discarded or fed to less susceptible species. Inspection of fields before harvest provides the best chance of detecting DHPA-producing plants.

Even though most DHPA-producing plants are not highly palatable, it is recommended that plants be eliminated from pastures and ranges. However, this can be extremely difficult when some species are prolific producers of seed that can remain dormant in soil for many years. This is exacerbated when a plant is translocated into an ecosystem that is favourable to growth and does not include its natural competitors, i.e., weed spread. Biological control programmes are being pursued, using insects that destroy the host plant (for example see CSIRO Fact Sheet, 2006), and species-specific herbicide regimens have been developed for most plants. However, an integrated and adaptive pasture weed management programme should be initiated (Thorne *et al*., 2005).

14.5.7 Livestock-mediated presence of hepatotoxic pyrrolizidine alkaloids in the human food supply

Hepatotoxic pyrrolizidine alkaloids may enter the human food supply when domestic livestock graze pastures and rangelands where DHPA-producing plants grow, or they are given fodder or prepared feed contaminated by DHPA-producing plants. In these cases, the animal-derived meat, offal (Seawright, 1994; FSANZ, 2001b; Fletcher *et al*., 2011) and milk (Schoental, 1959; Dickinson *et al*., 1976; Molyneux and James, 1990) can contain DHPAs. Layer hens that were inadvertently poisoned by DHPAs in the grain component of their diet have been shown to produce eggs containing DHPAs (Edgar and Smith, 2000).

For livestock-derived products such as milk, eggs and meat, it is not expected that humans will be exposed to the high levels of DHPAs found in contaminated grains (Steyn, 1933, 1935; IPCS, 1988; Gaul *et al*., 1994) or DHPA-containing herbal teas (Stuart and Bras, 1957; Bras *et al*., 1961; Brooks *et al*., 1970). This expected very low-level and/or intermittent exposure to dietary DHPAs can be predicted to produce quite different clinical signs in humans (Edgar *et al*., 2011). Instead of the haemorrhagic necrosis, rapidly developing abdominal ascites and veno-occlusive disease (or sinusoidal obstruction syndrome) that are associated with the higher levels of exposure, intermittent or long-term, low-level, exposure to DHPAs allows liver damage to repair between insults. Consequently, DHPA-related damage in the lung vascular system leading to pulmonary arterial hypertension and right heart congestive failure may become the dominant expression of intermittent or low-level exposure to DHPA (see Györika and Stricker, 2009). This is because such damage in the lungs, considered to be caused by DHPA 'pyrrolic' metabolites generated in hepatocytes escaping to the lungs, is likely to be less able to undergo repair.

The effects of DHPAs on livestock, the implications that these observations have for human exposure, and the responses of food safety authorities around the world have been reviewed by Molyneux *et al*. (2011) and Edgar *et al*. (2011).

14.6 Conclusions and future trends

Natural hepatotoxins abound in the animal feed and human food supplies. Acute effects are relatively easy to recognize and attribute to exposure to such natural toxicants and are relatively easy to quantify in terms of health effects and production losses. However, the chronic effects of long-term, low-level exposure to natural hepatotoxins may indeed be the 'iceberg below the waterline', especially with debilitating disease of unknown aetiology in humans and with production losses in livestock agriculture.

Many of the natural hepatotoxins are restricted in their geography or ecology and, while presenting significant local problems, do not present a significant global issue. On the other hand, the DHPAs and other hepatotoxins such as aflatoxins, by virtue of their wide distribution and proven capacity to contaminate human food and animal feed, have the potential to more broadly affect animals and people downstream from the farm.

There is no doubt that high-level exposure and even some chronic medium to low-level dietary exposures to DHPAs cause adverse effects in livestock and humans. DHPA-related toxicity due to intermittent consumption of DHPA-contaminated honey, bee-pollen, milk, eggs and grain is currently difficult to diagnose due to the slow, progressive development of liver and lung damage and cancer. Therefore, dietary DHPAs need to be actively considered as a contributing factor in all cases of cirrhosis, pulmonary arterial hypertension and cancer of undetermined aetiology.

In order to develop a clearer and more accurate understanding of the contribution to the cause of chronic disease and productivity losses by dietary hepatotoxins, the diagnosis of exposure needs to be improved. For the hepatotoxic PAs in particular, as an example, this will include making clinicians aware of a potential DHPA aetiology, developing new analytical techniques for specific biomarkers of exposure to DHPAs and/or any consequent developing disease state, and utilizing current and new analytical technologies to better define the extent of dietary exposure to the DHPAs.

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15

Feed additives and veterinary drugs as contaminants in animal feed – the problem of cross-contamination during feed production

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Abstract: This chapter explores the area of feed cross-contamination during the production stage at the feed mill, and includes a number of studies and models assessing exposure of non-target animal species and the transfer of residues. First, an introduction is given to the licensing of substances for inclusion in feed, including feed additives and veterinary drugs. This is followed by a description of recent developments in legislation, including new maximum levels for carry-over of coccidiostats into feed for non-target animal species arising from unavoidable cross-contamination and the potential hazards arising for animals and consumers of food from animal origin. The chapter closes with recent developments in analytical methods for feed and future perspectives.

Key words: feed additives, veterinary drugs, cross-contamination, eggs, meat, feed.

15.1 Introduction

The use of antibiotics in livestock to promote growth and to prevent bacterial infections can be traced to the 1940s when Jukes and co-workers carried out the initial experiments with chlortetracycline, obtained from a soil-based fungus called *Streptomyces aureofaciens* which was added to animal feed (Jukes 1985). The compound was found to effectively control disease in animals, but also, more unexpectedly, to enhance growth in poultry, pigs and other species. In the succeeding years, the use of other antibiotic compounds such as oxytetracycline, streptomycin and penicillin permitted the intensification of livestock rearing, with lower economic costs incurred due to infectious disease. In 1953, the UK government

introduced the Therapeutic Substances Act, which permitted the inclusion of penicillin and chlortetracycline in animal diets, but not exceeding a stated maximum of 100 g per ton of feed (cited from Lawrence and Fowler 2002).

The intensive use of medicated animal feeds raised in turn serious questions as to the impact on human health, and consequently their usage was regulated by Directive 70/524/EEC (EC 1970a), replaced in the meanwhile by EC Regulation 1831/2003/EC. In the UK, similar legislation was enacted in the form of the Medicines Act (Anon. 1968). As part of this act, the UK government had appointed a Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, also known as the Swann Committee (1968–1973). This committee recommended the ban on human therapeutic antibiotics as animal growth promoters in 1969. The committee concluded that 'the administration of antibiotics to farm livestock, particularly at sub-therapeutic levels, poses certain hazards to human and animal health', in particular the rise of resistance in enteric bacteria. The report by this committee, the Swann Report (Anon. 1969), has become one of the most influential publications on the area of veterinary use of antibiotics and the consequences for human health. Nearly 30 years later, the World Health Organisation convened a meeting on the subject in 1997 (WHO 1997) and determined that 'Antimicrobial use leads to the selection of resistant forms of bacteria in the ecosystem of use'. As part of the recommendations published from this meeting, it was suggested that the use of any antimicrobial agent as a growth promoter in animals should be terminated if the antibiotic is essential as a human therapeutic, or if there is evidence for the selection of cross-resistance to antimicrobials in human medicine. This finally resulted in the phasing out of all antimicrobial feed additives used as growth promoting agents in 2006. However, the use of licensed veterinary medicinal products, which are presented as premixes intended for mixing with feed for therapeutic purposes, is still in place to treat large numbers of animals, following a diagnosis and prescription by a veterinarian. The production of these medicated feeds occurs at commercial feed mills. Due to residual amounts of feeds that remain in the production lines (circuit), a certain degree of cross-contamination of subsequently produced feed batches seems technically unavoidable and raises the question to what extent these residual amounts pose a risk for animal and human health.

15.2 Regulatory provisions

In the European Union, feed additives cannot be marketed unless authorised under Council Regulation 1831/2003 (EU 2003). The primary scientific evaluation is conducted by the FEEDAP of the European Food Safety Authority (EFSA) assisted by the European Union Reference Laboratory for Feed Additives. This scientific evaluation may include the setting of maximum residue limits (MRLs) and follow-up regulatory control if

required. Authorised additives in feeding stuffs containing provisional and permanent authorisations under the old Directive 70/524 (EC 1970a) are listed in the Community Register of Feed Additives. A number of veterinary medicinal products (VMPs) listed under EC Directive 37/2010 (EU 2010) may be incorporated in medicated feed (MF). However, they require a veterinary prescription and only licensed premixes can be used. An example for a class of medication that falls into both categories is antimicrobials that are used for the prevention and therapy of coccidiosis in poultry and other animal species, such as rabbits, calves, lambs and piglets. A limited number of anticoccidial agents are approved for veterinary use. These are listed in the Annex of Allowed Substances in Commission Regulation No. 37/2010 (EU 2010). They are Amprolium, Decoquinate, Diclazuril, Halofuginone, Imidocarb, Lasalocid and Toltrazuril. The use of these coccidiostats as veterinary medicines allows for the incidence where there is a sporadic coccidiosis outbreak which may occur if there are no coccidiostats in the feed, or in the case of development of resistance, or even when the use of a vaccine is ineffective. This annex contains information on marker residues, animal species in which the substance is authorised, maximum residue limits, target tissues, therapeutic classification and other provisions according to Article 14(7) of Regulation (EC) No. 470/2009 (EU 2009a).

In addition, Commission Directive 2009/8/EC (EU 2009a) sets maximum content levels for 11 coccidiostats (compounds used for the prevention of coccidiosis) in feed, including Lasalocid sodium, Narasin, Salinomycin sodium, Monensin sodium, Semduramicin sodium, Maduramicin ammonium alpha, Robenidine hydrochloride, Decoquinate, Halofuginone hydrobromide, Nicarbazin and Diclazuril. These maximum levels are set in mg kg^{-1} (ppm) relative to a feeding stuff with a moisture content of 12% (Table 15.1) and aim at avoiding overexposure of the animals, as many of these compounds have a rather small safety margin and a slightly higher concentration in feeds may cause adverse effects even in target animal species.

In relation to these coccidiostats there has been a continued improvement in the sensitivity and substance coverage of analytical methods since the early 2000s with the development of more sensitive analytical methods in laboratories. As a result, there have been increasing reports of residues of these coccidiostats being detected in eggs and meat. It has been recognised by regulatory authorities that these residues are caused by unavoidable cross-contamination of feed batches during manufacturing. In response, legislation has been implemented, namely Commission Directive 2009/8/EC (EU 2009a), laying down the maximum levels of unavoidable transfer of coccidiostats into non-target feed, taking into consideration that feed business operators may produce within one establishment a broad range of feeds, resulting in different types of products being manufactured on the same production line and unavoidable cross-contamination from one feed

Table 15.1 Maximum content levels for coccidiostats in feed as laid down in Commission Directive 2009/8/EC and Council Regulation 2010/37/EC

Feed additive	Feed dose (mg/kg)	Non-target feed (mg/kg)		Withdrawal feed (mg/kg)
		Sensitive	Other species	
Lasalocid sodium	125	1.25 D, C, R, E, DA, LB, T > 12 wks, CL > 16 wks	3.75	1.25 CF, CL < 16 wks, T < 12 wks
Narasin	70	0.7 T, R, E, LB, CL > 16 wks	2.1	0.7 CF
Salinomycin sodium	70	0.7 E, T, LB, CL > 12 wks	2.1	0.7 CF, CL < 12 wks, RF
Monensin sodium	125	1.25 E, D, SR, Du, B, DC, LB, CL, T > 16 wks	3.75	1.25 CF, CL, T < 16 wks
Semduramicin sodium	25	0.25 LB, CL > 16 wks	0.75	0.25 CF
Maduramicin NH_4 alpha	5	0.05 E, R, T, LB, CL > 16 wks	0.15	0.05 CF, T < 16 wks
Robenidine HCl	70	0.7 LB, CL > 16 wks	2.1	0.7 CF, RFB, T
Decoquinate	40	0.4 LB, CL > 16 wks	1.2	0.4 CF
Halofuginone HBr	3	0.03 LB, CL > 16 wks, T > 12 wks	0.09 Except CL < 16 wks	0.03 CF, T < 12 wks
Nicarbazin	50	0.5 E, LB, CL > 16 wks	1.5	0.5 CF
Diclazuril	1	0.01 LB, CL > 16 wks, TF > 12 wks	0.03 Except CL < 16 wks, CF, TF < 12 wks	0.01 RFB

Key: D = dogs, C = calves, R = rabbits, E = equine, DA = dairy animals, LB = laying birds, T = turkeys, TF = turkeys for fattening, CL = chickens reared for laying, CF = chickens for fattening, RF = rabbits for fattening, RFB = rabbits for fattening and breeding, SR = small ruminants (sheep and goats), Du = ducks, B = bovine, DC = dairy cattle.

product to another. This directive amends Annex 1 of Directive 2002/32/EC (EU 2002a) on undesirable substances in animal feed. Commission Regulation No. 124/2009 (EU 2009b) works in tandem with 2009/8/EC setting maximum levels for the presence of these 11 coccidiostats in food resulting from the unavoidable carryover of these substances in non-target feed (Table 15.2).

15.2.1 Sampling and official inspection

Legislation concerning sampling and methods for the official control of feed is outlined in 70/373/EEC (EC 1970b). Methods for the sampling and analysis have since been updated through several pieces of legislation. Community methods of sampling for the official control are outlined in CD 76/371 (EC 1976). General rules for methods of analysis are outlined in CD 81/680/EEC (EC 1981). Some more specific legislation establishing reference methods for measurement of feed additives by HPLC are laid down in CD 93/28/EC (EC 1993), CD 93/117/EC (EU 1993), CD 1999/27/EC (EU 1999b) and CD 1999/76/EC (EU 1999a). Council Directive 95/53/EC (EU 1995) provides a framework for the development of official inspections in animal nutrition. This legislation is supplemented by Commission Directive 98/68/EC (EU 1998) containing information regarding standard documentation and checks to be performed on imports of feeding stuffs from third countries. The commission recommends that member states carry out a coordinated annual official inspection programme annually based on previous inspection results.

15.2.2 Maximum residue levels and analytical controls

Enforcement of these regulations and the introduction of maximum residue levels in target animal and non-target animal feeds requires the establishment of fit-for-purpose analytical methods. Commission Regulation (EC) 429/2008 (EU 2008) describes the requirements for analytical methods for assessment of feed additives. It differentiates methods of analysis for the active substance, methods of analysis for the determination of residues of the additive or of its metabolites in food, and methods of analysis relating to the identity and characterisation of the additive. In the case of the active substance the analytical method must be characterised according to Annex III of Regulation (EC) No. 882/2004 (EU 2004). Determination of the residues of the additive or its metabolite in food is more demanding and requires also Commission Decision 2002/657/EC to be taken into consideration (EU 2002b). This is a comprehensive document concerning the performance of both screening and confirmatory analytical methods and interpretation of results for the monitoring of residues in live animals and animal products.

Table 15.2 Maximum content levels for coccidiostats in eggs as laid down in Commission Regulation 2009/124/EC and Council Regulation 2010/37/EC

Substance	Species	Maximum residue limits in foodstuffs of animal origin (μg kg^{-1})						
		Eggs	Milk	Liver	Kidney	Muscle	Skin and fat	Other
Lasalocid sodium	Py	150		100	50	20	100	
	NT		1	50	50	5	5	
Narasin	CF			50	50	50	50	
	NT	2	1	50	5	5	5	5
Salinomycin sodium	CF			5	5	5	5	
	RF							
	NT	3	2	5	2	2	2	2
Monensin sodium	CF			**8**	**8**	**8**	**25**	
	Turkeys			**8**	**8**	**8**	**25**	
	Bovine		2	30	2	2	10 (*fat only*)	
	NT	2	2	8	2	2	2	2
Semduramicin	CF							
	NT	2	2	2	2	2	2	2
Maduramicin	CF							
	Turkeys							
	NT	2	2	2	2	2	2	2
Robenidine	CF			800	350	200	1300	
	Turkeys			400	200	200	400	
	RFB							
	NT	25	5	50	50	50	50	5
Decoquinate	CF							
	Bovine*							
	Ovine*							
	NT	20	20	20	20	20	20	20
Halofuginone	CF							
	Turkeys							
	Bovine*			30	30	10	25 (*fat only*)	
	NT	6	1	30	30	3	3	3
Nicarbazin	CF			15000	6000	4000	4000	
	NT	100	5	100	100	25	25	25
Diclazuril	CF			1500	1000	500	500	
	TF			1500	1000	500	500	
	RBF			2500	1000	150	300	
	Porcine							
	Ruminants							
	NT	2	5	4	40	5	5	5
Amprolium	Poultry							
Imidocarb	Bovine		50	2000	1500	300	50	
	Ovine*			2000	1500	300	50	
Toltrazuril	AMFPS*			500	250	100	150	
	Poultry**			600	400	100	200	

Bold print indicates a provisional MRL.
* Not for use in animals from which milk is produced for human consumption.
** Not for use in animals from which eggs are produced for human consumption.
Key: Py = poultry, CF = chickens for fattening, RF = rabbits for fattening, RFB = rabbits for fattening and breeding, TF = turkeys for fattening, AMFPS = all mammalian food-producing species, NT = non-target.

15.3 Cross-contamination at feed mills

The production facility from which feedstuff originates presents a tangible point of possible cross-contamination. One possible source for contamination of non-medicated feedstuff with medicated rations is the manner in which it is produced and managed at the feed mill, where it is likely that within a short period of time, both medicated and non-medicated batches will be milled and pelleted. Kennedy *et al*. (1998b) explored this issue in a study on carry-over at a feed mill with monensin. The authors found that monensin was present in non-medicated broiler rations at levels in excess of 5% of the therapeutic dose (110 mg kg^{-1}) in 22.5% of 40 withdrawal feeds. The pre-pelleting bins, and the pelleting die, were identified as the most likely reservoirs of contamination, and feed handling at the mill was changed accordingly. Subsequently, the number of feed batches containing more than 5% of the therapeutic dose of monensin dropped from 22.5% to 2.5%.

Incidences of lasalocid residue in eggs in Northern Ireland were also attributed to carry-over at the feed mill (Kennedy *et al*. 1996). As part of the investigation, laying hens were given feed containing lasalocid at concentrations analogous to those expected as a consequence of carry-over at the feed mill (between 0.1 and 5.0 mg kg^{-1}). The levels of lasalocid subsequently found in the eggs produced by these birds were comparable to the quantities found in eggs as part of a survey of lasalocid in eggs in Northern Ireland. In this survey, 66% of eggs tested contained residues in excess of 0.3 μg kg^{-1}. The authors noted that lasalocid residues persisted in eggs for 10 days after withdrawal of the medicated feed, indicative for the long elimination period and the persistent presence of residues in eggs sold to consumers. Similar work on the excretion of halofuginone with eggs following the administration of contaminated feed was performed by the same group (Yakkundi *et al*. 2002). These investigations demonstrated that a contamination rate of 0.1 mg kg^{-1} (3% of the therapeutic dose of halofuginone) resulted in contamination of eggs at a concentration in excess of 6 μg kg^{-1}.

The possible mechanisms by which cross-contamination at a feed mill may occur were explored by McEvoy *et al*. (2003). The authors examined three sequential batches of 3 tonnes each, of nicarbazin-free feed produced after a batch which had contained nicarbazin at the statutory level of 125 mg kg^{-1}. Sampling was performed both before and after pelleting of the feed. It was observed that in the first nicarbazin-free batch produced after the nicarbazin-containing ration, a level of nicarbazin of 3.4 mg kg^{-1} was found prior to pelleting. Higher concentrations were found post-pelleting – after 8 tonnes had passed through – and up to 7.2 mg kg^{-1} were determined in the feed. The primary cause of this high level of contamination was determined to be the practice of returning post-press sieved material into the pre-press bins. The suggested intervention strategy in this instance

was a better segregation of feedstuff, according to whether it contained nicarbazin or not. Finally, the potential for low levels of coccidiostat contamination originating at the feed mill to lead to detectable residues in eggs was described by Daeseleire *et al*. (2006) through an extensive kinetic depletion study.

While these first experiments addressed the carry-over of coccidiostats, licensed in Europe as feed additives, the carry-over of other veterinary pharmaceuticals has been investigated also. McEvoy (2002) describes a study on the carry-over of the sulphonamide sulphadiazine. The author found that following the manufacture of a medicated batch of feed (125 mg kg^{-1}), a low level of carry-over was observed for the next 12 tonnes of feed milled (~2 mg kg^{-1}). McCracken *et al*. (1997) demonstrated in earlier work that for the nitrofuran antibiotic furazolidone a considerable carry-over at a feed mill has to be expected as well. Following production of a medicated batch of feed (353 mg kg^{-1}), a level of furazolidone of 12.7 mg kg^{-1} could be detected in the next batch produced. However, by the third batch, no further furazolidone could be detected. Subsequent investigations of feed contamination with nitrofurans at the point of milling are now redundant, due to the ban on their use in food-producing animals.

15.4 On-farm cross-contamination

The origin of contamination of feedstuff may be located at any point along the production and distribution chain, from the point of manufacture at the feed mill as discussed in the previous section, to the stage of administration and use on a farm. For example, survey work carried out by Cannavan *et al*. (2000) established the presence of residues of nicarbazin (in the form of 4,4 -dinitrocarbanilide (DNC)) in 39 of 190 eggs tested. The authors correlated the quantity of DNC (and the 4,6-dimethyl-2-hydroxypyrimidine (DHP) constituent of nicarbazin) found in the eggs to the quantity of the drug administered in the feed and concluded that nicarbazin at levels greater than 2 mg kg^{-1} leads to occurrences of residues of the drug in eggs at levels greater than the UK differential action limit (DAL) of 100 μg kg^{-1}. At the time of writing, nicarbazin was authorised in the EU for use in broiler chickens, but was contraindicated for laying hens. Hence, the authors attributed the observed occurrence of nicarbazin residues in egg to a contamination of feed at the mill. However, additional work by the same group of authors demonstrated that cross-contamination of feed with nicarbazin can be a consequence of how the chickens are housed (Cannavan and Kennedy 2000). When animals were housed on deep litter, nicarbazin was excreted with the faeces and taken up again by the animals over a longer period. This phenomenon was not observed when animals were kept in cages (with wire flooring), confirming the assumption of recycling within the animal facilities. This is of concern, as violative levels of DNC may occur

even when nicarbazin-free feed was supplied only in replacers (non-laying young hens) and the appropriate withdrawal period was observed.

O'Keeffe *et al*. (2007) also found poor management of feed supply on-farm was resulting in violative levels of nicarbazin residues in broiler liver. Levels of DNC in the range 20–1600 μg kg^{-1} were observed in broilers scheduled as being withdrawn for longer than six days. Issues identified as particularly problematic included the failure to empty and clean feed bins before delivery of nicarbazin-free feed. The authors produced an 11-point plan to assist poultry farmers in avoiding nicarbazin residues (Fig. 15.1).

While coccidiostat contamination of feedstuffs remains a significant issue, issues of cross-contamination of medicated feedstuffs at the farm level containing antibiotic drugs have also been observed and reported. In work performed by Rea (1993), high incidences of sulfa drug contamination in swine tissue were attributed to, among other things, cross-contamination of non-medicated withdrawal feed from on-farm mixing and handling. Other causes were a failure to observe the indicated withdrawal period and lagoon water recycling. Sulfa-drugs pose particular problems in this respect, as even relatively low levels of cross-contamination (~2 mg kg^{-1} feed) of non-medicated feed with sulphamethazine can lead to residues in porcine tissue (McEvoy 2002). It was noted that this does not necessarily apply to all sulphonamides, as for example sulphadiazine, due to its lower rate of absorption after oral application with feed, will not result in detectable residues in pigs.

15.5 Transfer of residues to food

15.5.1 Horizontal transfer and residue depletion of veterinary medicinal products in broiler breeders

A transfer from parent broiler breeders to progeny broilers was demonstrated for the nitrofuran group of drugs (McCracken *et al*. 2005). Broiler breeders were given medicated feed containing 400 mg kg^{-1} nitrofurazone, nitrofurantoin, furazolidone or furaltadone. After a week of treatment, eggs collected contained concentrations of the side-chain metabolites of the nitrofuran compounds up to 1567 μg kg^{-1}. The livers of one-day-old chicks hatched from these eggs were then tested for nitrofuran metabolites. For the metabolites AHD, AOZ and AMOZ, liver concentrations were relatively low (<12 μg kg^{-1}). For SEM, a level of 26.6 μg kg^{-1} was observed. Collectively, the work demonstrated that treatment of parent animals with nitrofurans could lead to detectable quantities of side-chain metabolites being present in the progeny. It needs to be reiterated that the entire class of nitrofurans is banned currently for use in food-producing animals, including avian species.

Slight differences in physico-chemical properties between compounds can result in different behaviour, in terms of accumulation in a matrix and

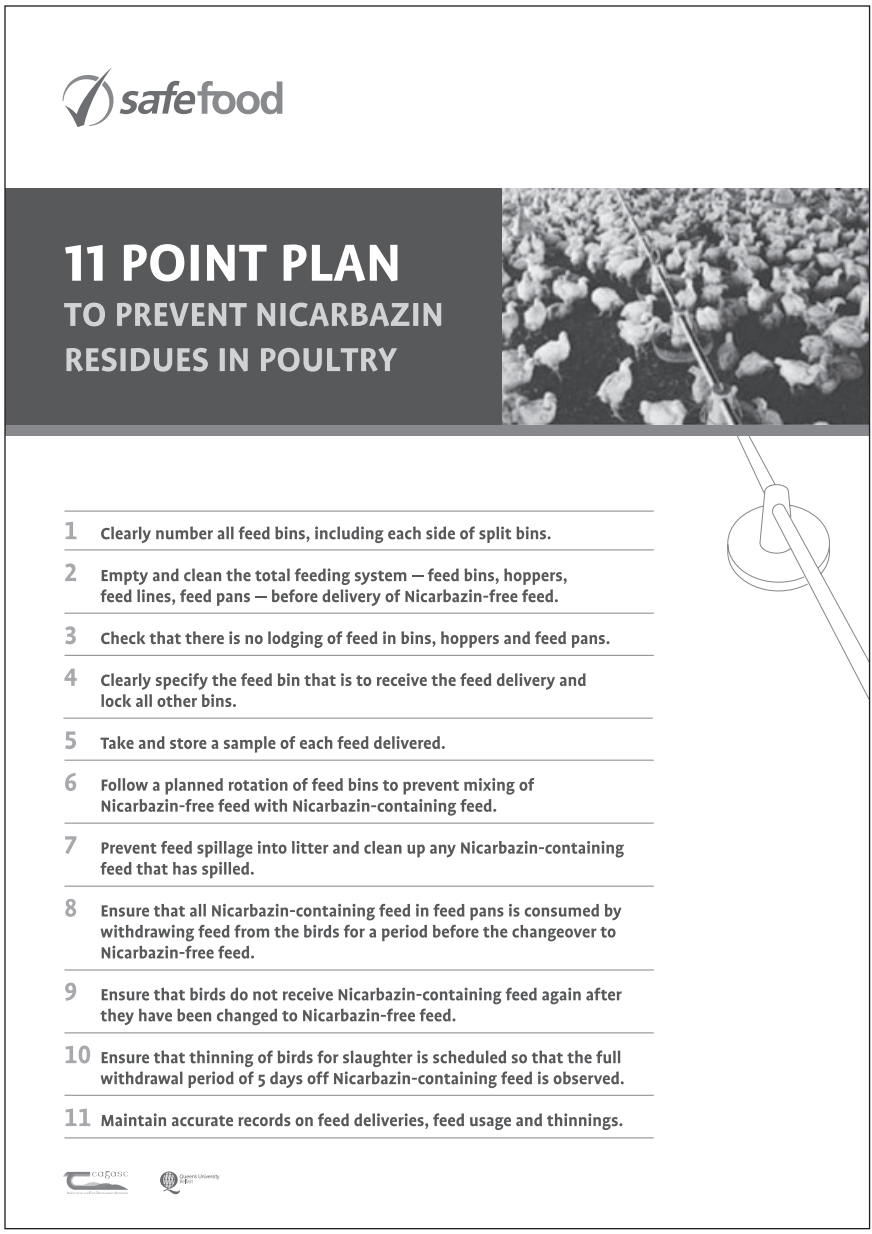

Fig. 15.1 Poster with an 11-point plan to prevent nicarbazin residues in poultry (O'Keeffe *et al*. 2007).

rate of depletion from animal tissues. It has been noted that ionophoric coccidiostats exhibit significant differences in their capacity to accumulate in poultry meat and eggs (Kennedy *et al*. 1998b). In work by Kennedy and colleagues (1995a), broiler breeders were given feed containing a 90 mg kg^{-1} of lasalocid and residual concentrations in liver were monitored over a seven-day period, using an ELISA method. By the end of this period, the concentration of the lasalocid had decreased to 10 μg kg^{-1}. A similar study was performed with salinomycin, wherein broilers were fed a medicated feed containing a 60 mg kg^{-1} dose of the drug (Kennedy *et al*. 1995b). The authors observed that here, only very small quantities of the drug could be determined in liver and muscle, and that within two days of withdrawal of the medicated feed, residues were below the decision limit of the ELISA employed (0.31 μg kg^{-1} for muscle, 0.29 μg kg^{-1} for liver). Crooks *et al*. (1997) investigated the depletion of monensin from poultry liver, applying also an ELISA method. Monensin was below the limit of detection of the assay (2.91 μg kg^{-1}) within three days after withdrawal of the medicated feed. Diclazuril appears to be somewhat more persistent in tissue matrices. In work by Mortier *et al*. (2005a), following administration of feed containing 730 μg kg^{-1} diclazuril for 10 days, the steady-state concentration of diclazuril in liver was determined to be 722 μg kg^{-1}. Nine days after withdrawal, diclazuril was still present in the samples tested, but at levels below the established MRL.

While all three studies described above used an ELISA for residue testing, similar analyses were conducted using chemical assays (Kennedy *et al*. 1998a). With this study, a survey of the content of ionophore residues in eggs in Northern Ireland was performed (161 eggs surveyed). The authors determined that lasalocid had a 520-fold greater capacity to enter into and accumulate within eggs, as compared to monensin (Fig. 15.2). Lasalocid had a 19-fold greater capacity to accumulate in egg than salinomycin. Thus, it is evident that lasalocid possesses the greatest potential to cause residues in eggs. The capacity of eggs to act as drug residue 'storage depots' was explored in work performed by Donoghue and Myers (2000), using the human drug magnevist as a model compound. Employing a magnetic resonance imaging technique, the authors could image the accumulation of the drug in eggs laid by hens following injection, noticing a ring-like deposition mechanism. Although this work used an injection of a drug rather medicated feed, the accumulation of certain drugs in preovulatory egg yolks explains the long excretion times even after withdrawal of the drug under consideration.

Furusawa (2001) has performed an investigation into the transfer of 12 different veterinary drugs into eggs under controlled conditions. The compounds were added to feed for laying hens over a period of 14 days, at a concentration of 500 mg kg^{-1}. The concentrations of the drugs were then determined at two-day intervals after the start of feeding. It was noted that a plateau concentration was reached for oxytetracycline, tylosin, furazolidone and all sulfonamides in four days. Chloramphenicol,

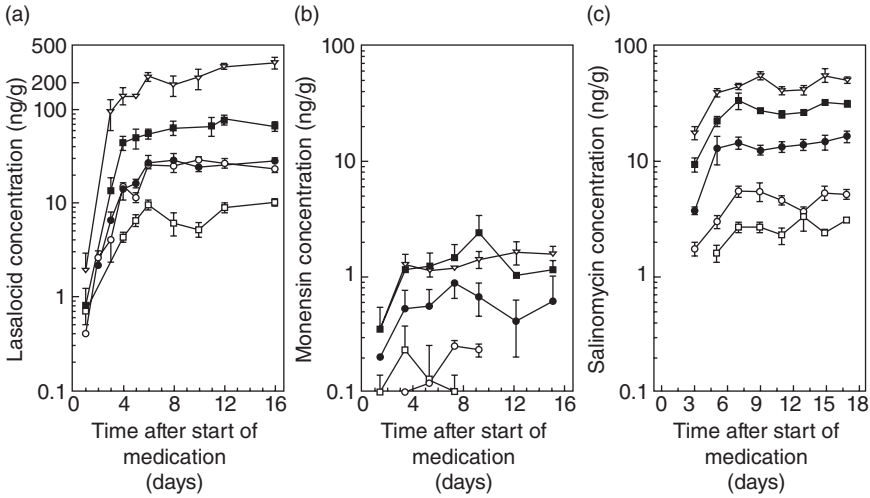

Fig. 15.2 Accumulation of lasalocid, monensin and salinomycin in eggs collected from birds fed commercial diets containing them at contamination levels of (a) lasalocid at □, 0.1; ○, 0.3; ●, 0.5; ■, 1.0; ∇, 5.0 mg kg^{-1}; (b) monensin at □, 1.1; ○, 1.8; ●, 4.6; ■, 8.0; ∇, 12.9 mg kg^{-1}; (c) salinomycin at □, 0.9; ○, 1.8; ●, 4.6; ■, 9.1; ∇, 13.9 mg kg^{-1} (Kennedy *et al*. 1996, 1998).

amprolium, nicarbazin and ormetoprium reached a constant level after six days. No bacitracin or chlortetracycline could be detected in the egg samples. Overall, the author determined transfer rates of between 0.005% for tylosin and 1.54% for sulfadimidine.

Depletion data for various coccidiostats into egg were also investigated in detail by Mortier *et al*. (2005b). Laying hens were given feed containing various coccidiostats, ranging from 1 mg kg^{-1} for diclazuril up to 40 mg kg^{-1} for narasin and nicarbazin (corresponding to the statutory levels). The longest withdrawal periods observed were for robenidine (26 days' withdrawal required to obtain a negative sample via LC-MS/MS) and nicarbazin (23 days). Depletion studies on the coccidiostats toltrazuril and halofuginone were described by Mulder and co-workers (2005), showing that these drugs (and ponazuril, a metabolite of toltrazuril) preferentially partitioned into the yolk. Chickens given a medicated feed containing 3 mg kg^{-1} halofuginone exhibited a maximum egg concentration of 450 μg kg^{-1}, with rapid depletion once treatment was terminated. Residue levels dropped below the 2 μg kg^{-1} limit of detection (via LC-MS/MS) after 12 days' withdrawal. With toltrazuril, it was found that the metabolite ponazuril was the dominant residue found. Following administration of drinking water containing 78 mg L^{-1} for two days (repeated after a five-day interval for another two days), ponazuril concentration in egg increased to 11000 μg kg^{-1}. Nineteen days post-treatment, the level of toltrazuril had dropped below the limit of detection of 30 mg kg^{-1}, but ponazuril was still present in quantities of up

to 1600 μg kg^{-1}. Work by Cannavan and Kennedy (1997) investigated the transfer of a nitroimidazole antibiotic into egg; following addition of dimetridazole (DMZ) to the diet of chickens at a concentration of 10 mg kg^{-1}, measurable quantities of DMZ could be determined after one day. The mean concentration of DMZ in eggs taken after seven days was 21.6 μg kg^{-1} (five nitro-imidazoles, including DMZ, are banned for use in food-producing animals in Europe).

15.5.2 Horizontal transfer and residues in dairy milk

Only few papers have been published addressing the transfer of residues into milk following the use of medicated feeds. Kaneene and Ahl (1987) carried out an epidemiological evaluation using statistical analysis to determine whether management and attitudinal factors significantly influenced the occurrence of residues in dairy herds. Their findings indicated that use of medicated feed was one significant factor in residue occurrence. It was found that occurrence of residues increased with increased frequency of use and surprisingly that residues had a higher incidence on farms that used commercial medicated feeds than those that mixed their own feed. This was attributed to greater awareness of withdrawal times but also to the fact that the use of commercial medicated feeds is more prevalent in larger farms with increased numbers of employees. McEvoy *et al*. completed two separate studies on the transfer of sulphamethazine and chortetracyline from contaminated feed to cow's milk (McEvoy *et al*. 1999, 2000). Both studies contained a control group, low and medium dosage groups representative of the contamination concentrations that may be present in non-medicated feedstuffs, and a high dosage group which represents what might happen if two or three times the top therapeutic doses for pigs (300 mg chlortetracycline kg^{-1}, 100 mg sulphamethazine kg^{-1}) were added to the wrong ration. The authors concluded that when residues of tetracycline are detected in milk, feed contamination may be eliminated as the probable cause. In the case of sulphamethiazine the study suggested that in feedmills the manufacturing of dairy compound feedstuffs could be scheduled directly after the manufacture of medicated pig rations. Van Rhijn *et al*. (2000) investigated the excretion of nicarbazin into dairy milk after administration of feeds containing nicarbazin at concentrations of 1, 5 and 12.5 mg kg^{-1}. Some body fat samples were also analysed after the completion of the study. No nicarbazin was detected by the authors, indicating that carry-over of nicarbazin form feed to milk is not a very likely process. Similarly, Bagg *et al*. (2005) was unable to find detectable monensin residue in lactating Holsteins receiving up to 4865 mg monensin per day.

15.6 Recent developments in analytical methods

Historically, reversed phase liquid chromatography with spectrophotometric detection was the preferred method of analysis for feed additives,

including coccidiostats. These methods generally have extended run times and are specific to single substances or substance groups. Today, the majority of established chemical assays look to exploit liquid chromatography coupled to tandem mass spectrometry. This technology allows for the development of multi-residue methods with enhanced sensitivity and shortened analysis times. Again, methods of analysis for coccidiostats are a prominent example to illustrate these developments.

15.6.1 Reference methods for determination of coccidiostats in feed

Several reference methods are available for detecting individual or multiple coccidiostats in feed. These reference methods include methods that have been submitted as part of the registration process, AOAC Official Methods (AOAC 1990) or EN (European standard methods). The ionophores, namely monensin, salinomycin and narasin, may be simultaneously detected by HPLC-UV (AOAC Official Method 2006.01 Monensin, Narasin and Salinomycin in Mineral Premixes, Supplements and Animal Feeds). The quantification range of the method ranges from 1 mg kg^{-1} to 200 g kg^{-1}. Feed samples are extracted with a mixture of methanol and water (90:10, v/v) with mechanical shaking for 1 h, followed by filtration and dilution of the extract. The three ionophores are detected by HPLC-UV at 520 nm following post-column derivatisation with vanillin.

An HPLC method for detecting the chemical coccidiostat decoquinate was published in recent years (AOAC Official Method 2008.08 Decoquinate in Animal Feeds). This method requires an initial extraction step with 1% calcium chloride–methanol solution, with mechanical shaking for 90 min. Following centrifugation and dilution, decoquinate is analysed by HPLC with fluorescence detection. Suspect positives in non-target feeds can then be confirmed by using an alternative excitation wavelength. The limit of quantification (LOQ) for the method was 0.5 mg kg^{-1}. A method for the detection of the polyether antibiotic lasalocid has also been published (AOAC Official Method 2008.01 Lasalocid Sodium in Animal Mixes and Premixes), covering both inclusion levels and trace levels of this substance. Extraction is performed using 0.5% HCl-acidified methanol and analysed by HPLC. All five lasalocid homologues were detected via fluorescence detection, with excitation at 314 nm and emission at 418 nm. The limit of detection (LOD) for the method was 0.3 mg kg^{-1}, while the LOQ was 1 mg kg^{-1}.

15.6.2 LC-MS/MS methods for coccidiostats

Dai and Herrman (2010) have presented two individual LC-MS/MS platforms for quantification of monensin in animal feed and milk. The platforms investigated were an LC linear ion trap (LC-LIT) and an LC triple quadrupole (LC-QqQ). For sample preparation, 3 g of feed was extracted using 100 mL 90% methanol in water. The authors stated that the LC-QqQ

approach represented a more robust means of analysis. Although both methods could detect residual monensin in milk down to a level of 1 μg kg^{-1}, the authors observed that the triple quadrupole approach displayed lower matrix dependence, a lower limit of quantification, and better intermediate precision. Feedstuffs containing salinomycin at levels of 1, 5 and 9 mg kg^{-1} were tested using an LC-MS/MS method. The authors observed a strong matrix influence but LODs of 0.007 and 0.006 mg kg^{-1} were achievable in poultry and cattle feeds, respectively.

Olejnik *et al*. (2009) recently developed a comprehensive method for 12 coccidiostat residues from liver based on MeCN extraction with purification on neutral alumina and Oasis HLB cartridges. Other coccidiostats of interest include amprolium, imidocarb and toltrazuril (particularly toltrazuril sulphone) which are not addressed in either 2009/8/EC (EU 2009a) or 2009/124/EC (EU 2009b) but are authorised for use as veterinary medicines in the EU. Few multi-residue methods have been reported for these analytes as they present a number of challenges. In the case of amprolium, poor chromatographic retention is an issue, and for toltrazuril there is difficulty in producing sufficient confirmatory ions in the electrospray ionisation. Kinsella *et al*. (2009) reported that some groups have successfully measured toltrazuril residues in eggs. Hormazabal and Yndestad (2000) developed a complex LLP method for isolating coccidiostat residues including amprolium from tissue, plasma and egg. Amprolium ethopabate and ionophore extracts were successfully analysed using different LC-MS detection systems.

Vincent *et al*. (2008) reported a LC-MS/MS method for measuring six ionophores (maduramicin, semduramicin, narasin, salinomycin, monensin and lasalocid) in target feed. Following extraction of samples with a mixture of methanol and water (90:10, v/v), extracts were purified on silica SPE cartridges. Validation experiments were executed at concentration levels of the target analytes of between 1 and 9 mg kg^{-1}. The same group later reported a method for detecting the same substances in non-targeted feed and at cross-contamination levels (Vincent *et al*. 2011). This method is based on an extraction with either acetonitrile or methyl isobutyl ketone, followed by centrifugation and filtration prior to LC-MS/MS analysis. LODs for the method ranged from 2 μg kg^{-1} (maduramicin in poultry feed) up to 60 μg kg^{-1} (lasalocid in pig feed).

A more comprehensive multi-residue method for coccidiostats in animal feed was described by Delahaut and co-workers (2010). Using LC-MS/MS, a method for the extraction, detection and confirmation of 11 coccidiostats referenced by Regulation 2009/8/EC (EU 2009a) was developed. Sample preparation involved extraction with 10% Na_2CO_3 solution and acetonitrile prior to LC-MS/MS analysis. Repeatability ranged between 0.9 and 13.6%. Cronly *et al*. (2011) also reported an assays for coccidiostats in feed a similar method, using sodium chloride and magnesium sulphate to improve recovery. A freezing-out step at –80°C was also included, to reduce the number of matrix interferents present in the samples. Precision values ranged from

4.4 up to 9.1% (relative standard deviations). Through these methods, the capacity to analyse multiple coccidiostats using ever more sophisticated sample preparation techniques continues to evolve.

15.6.3 Analytical methods for antibiotics and veterinary drugs

Historically, microbiological inhibition methods have been used as the standard methods for antibiotic substances (e.g. AOAC Method 957.23 – Antibiotics in Feeds, Microbiological Methods).

An HPLC method for the antibiotic avilamycin in poultry feeds was reported by Scott *et al*. (1999) that enables the detection of both biologically active substances, avilamycin A and B. Feed samples were cleaned up on a silica SPE cartridge, with elution via a mixture of acetone and dichloromethane. Separation was performed on a Kromasil C18 column with UV detection at 295 nm. The authors reported satisfactory recoveries (93.3–97.3%) for the method, with avilamycin factors in feed tested ranging from 4.45 to 17.82 μg g^{-1} for factor A and from 0.80 to 3.18 μg g^{-1} for factor B.

Macrolide antibiotics were investigated in feed by de la Huebra and coworkers (2010). Their method permits simultaneous detection of six different macrolides in feed via HPLC-electrochemical detection. Extraction was performed with methanol:water (1:1, v/v), followed by clean-up on acidic alumina and Oasis™ HLB SPE cartridges. Depending on the target analyte, relative standard deviations of 3.6% to 10.1% were observed.

Several multi-residue methods targeting veterinary drugs in animal feed have been published in the recent past. For example, a method for the detection of 11 quinolones in feed was proposed by Galarini *et al*. (2009). Samples are extracted with metaphosphoric acid:acetonitrile (70:30, v/v) and cleaned on Oasis™ HLB cartridges, followed by separation on a Gemini C18 column and detection using both UV and fluorescence. Limits of detection for the method were in the range of 0.04–0.8 mg kg^{-1}.

Shen *et al*. (2009) described a high-performance capillary electrophoretic method for the determination of five benzimidazoles in animal feeds. Compounds included were thiabendazole, albendazole, fenbendazole, mebendazole and oxfendazole. Following homogenisation, feed samples were extracted with ethyl acetate/1M Na_2CO_3 and mixed. Supernatant was removed following centrifugation and the extraction procedure was repeated. Extracts were dried down and reconstituted in ethylene glycol/ethanol/0.2M HCl. Following a further SPE clean-up step, samples were reconstituted in 400 mL of 5% formic acid in methanol, and analysed via capillary electrophoresis, with detection at 295 nm. The linear range for the method was 1–80 μg mL^{-1}, while in all cases RSDs were below 10%.

15.6.4 Detection of banned substances in feed

Since 1 January 2006, no antibiotics have been approved for use as growth promoters within the European Union (Castanon 2007), under Regulation

1831/2003 (EU 2003). Previously, substances such as avilamycin, flavophospholipol, salinomycin and monensin as well as bacitracin, spiramycin, tylosin, virginiamycin and olaquindox had been applied as growth-promoting agents in different animal species, particularly broilers and fattening pigs. The outright ban on the compounds within this category creates the need for vigilance and thorough, reliable analytical methods for monitoring.

A method developed by Van Poucke *et al*. (2003) detailed a means of extracting and detecting five banned antimicrobial growth promoters (zinc bacitracin, virginiamycin, spiramycin, tylosin and olaquindox) in feed, via SPE (on Oasis™ HLB cartridges) and LC-MS/MS analysis. MS-MS data was collected via multiple reaction monitoring (MRM) mode, with positive ionisation. The detection limits for the method are 0.8 mg kg^{-1} for zinc bacitracin and 0.5 mg kg^{-1} for the remaining compounds. A follow-up study on the effect of experimental design on method performance noted that the composition of the feed can affect what extraction conditions are required for optimal extraction. While some of these compounds (spiramycin, tylosin and virginiamycin) were preferentially extracted with 100% methanol, bacitracin and olaquindox gave more significant responses (in terms of peak area) with 50–70% methanol (v/v). An extraction solvent of 70% methanol (v/v) and 2% formic acid (v/v) was shown to give the best overall response. A survey performed in 2006 by several laboratories, which utilised this analytical method, revealed that the use of tylosin as a feed additive was continuing, at concentrations comparable to those typical of the banned antimicrobial growth promoters (Van Poucke *et al*. 2006).

In a review on residue analysis of veterinary drugs and growth-promoting agents, Stolker *et al*. (2007) highlight the intrinsic difficulties of developing analytical methods for feedstuffs – the heterogeneity of composition, inter-batch differences, and ion suppression during analysis. This may in part explain the paucity of sensitive analytical methods to date for salinomycin, avilamycin, flavophospholipol and monensin in feed. However, several groups have demonstrated that analytical detection and quantification of these compounds in feedstuffs is feasible.

The fragmentation of moenomycin antibiotics in mass spectrometric analysis was examined in detail by Eichhorn and Aga (2005). It was determined that because of the propensity of these molecules to form various sodium adduct ions in positive ion mode, the negative ion mode offered a more suitable (and sensitive) means of analysis. A method was published in 2007 by Pérez *et al*. (2007) to determine flavophospholipol (moenomycin-A) in chicken litter. Sample clean-up was achieved using a combination of pressurised liquid extraction (PLE) and SPE. Purified extracts were injected onto a C_{18} column and detected by an LC-MS/MS instrument, with both positive and negative ion detection feasible. A LOQ of 0.02 mg kg^{-1} was determined. The authors noted that due to the highly lipophilic nature of flavophospholipol, it was necessary to use a more non-polar C_4 stationary

phase for the SPE clean-up step, as well as methanol at 50°C during the PLE clean-up, to obtain satisfactory recoveries. Gallo and co-workers (2010) developed an alternative method for moenomycin A in feedstuff using LC-MS/MS, with a LOD of 0.1 μg g^{-1}. Feed (5 g) was extracted with 50 mL ammonium hydroxide 25%:methanol (1:9, v/v). The sample was vortexed, sonicated and mixed, prior to centrifugation, before clean-up on an Oasis™ HLB cartridge. Analysis was executed on an ion-trap LC-MS/MS system. The method was validated for a range of 0.5–30.0 g g^{-1}. Mean recoveries ranged from 83 to 94%.

5-Nitroimidazoles have been banned from use in animal feed due to concerns about their mutagenic and potential carcinogenic properties. Capitan-Vallvey and co-workers (2007) have developed a method for the simultaneous determination of six of these compounds in animal feed via a LC-MS method. The method was successfully applied to the determination of the 5-nitroimidazole compounds studied down to a concentration of 0.05 mg kg^{-1}.

Banned from use in food-producing animals in the European Union since the 1990s, nitrofurans are a group of antimicrobials that were widely used for the treatment of gastrointestinal infections in livestock, and as a growth promoter. The compounds were banned due to concerns about their potency as carcinogens and mutagens (Van Koten-Vermulen *et al*. 1993). However, despite the ban, there have been incidences of nitrofuran residues being found in commodities such as pork (O'Keeffe *et al*. 2004), indicating abuse of these drugs. An earlier method for determination of nitrofurans in feed premixes was presented by Moretain and co-workers (1979). More recently, a liquid chromatographic method for the determination of nitrofurans in feed was presented by Barbosa and co-workers (2007). In this work, both UV diode array and mass spectrometric means of detection were employed. Using the LC-UV method, the authors could simultaneously determine furaltadone, furazolidone, nitrofurantoin and nitrofurazone in feed samples down to levels of 50–100 μg kg^{-1}, although preferably as a screening method. The LC-MS/MS method proved more adept at low-level detection (20–50 mg kg^{-1}), as well as being more suitable for confirmatory analysis.

McCracken and Kennedy (1997) used both LC-UV and LC-MS to detect furazolidone residues in animal feed, following sample clean-up on an alumina column. A LOD of 1 mg kg^{-1} was achieved. Analysis of fish feed for furazolidone and its metabolite 3-amino-2-oxazolidinone has been described by Hu *et al*. (2007). The method again used both LC-UV and LC-MS/MS for separation and detection, with a LOD of 0.4 μg kg^{-1} for furazolidone. Simultaneous detection of the four main nitrofuran drugs in feed was described by Vinas *et al*. (2007). Detection was via UV spectroscopy, and limits of detection of 0.21–0.27 mg L^{-1} were attained. Analysis was applied to both poultry feed and farm water samples. However, despite these examples, most published methods for nitrofuran residue detection

have targeted their metabolites in tissue, which requires a different analytical approach (Conneely *et al*. 2002).

15.6.5 Multi-class assays

Aside from the targeting of compounds specifically banned under recent legislation, methods addressing the possibility of other antibiotics being present in feedstuffs continue to be developed, particularly multi-residue LC-MS/MS methods. De Alwis and Heller (2010) have published a method for the determination of 13 antibiotic compounds in distillers' grains, a by-product of ethanol production used for feed which may potentially contain antimicrobials that were used during the fermentation process. Analytes covered by the method included ampicillin, penicillin G, tetracycline, oxytetracycline, chlortetracycline, bacitracin A, virginiamycin M1, chloramphenicol, erythromycin A, clarithromycin, tylosin A, monensin A and streptomycin. Starting from a 5 g sample of distillers' grains, analytes were extracted with solutions of EDTA and trichloroacetic acid. Aliquots were cleaned up by SPE on either Oasis™ HLB cartridges or Isolute™ CBA cartridges. While most analytes were quantified using a phenyl column combined with ESI-MS/MS, streptomycin was quantified using a HILIC column and ACPI-MS/MS. Absolute recoveries for the method ranged from 50% to 100%.

Finally, a comprehensive 33-antibiotic method for the analysis of feed was published by Boscher and co-workers (2010). Detection was based on LC-MS/MS using both positive and negative modes. The scope of the method included tetracyclines, quinolones, penicillins, ionophore coccidiostats, macrolides, sulfonamides, quinoxalines, phenicols, lincosamides and others. Sample extraction was performed with a mixture of acetonitrile, methanol and McIlvaine buffer at pH 4.6, followed by a dispersive SPE clean-up. LOQs ranged from 3.8 μg kg^{-1} (lincomycin) to 65 μg kg^{-1} (bacitracin). RSDs ranged between 1.6% and 14.5% at the lowest spiking level for enrofloxacin and florfenicol, respectively.

15.6.6 Screening assays

LC-MS/MS methods of determining the presence of veterinary medicinal products in feedstuffs offer definitive means of confirmatory testing. However, auxiliary methods of screening large quantities of samples are also desirable, to improve overall sample throughput. In this section, we will examine some recent examples of screening methods targeting veterinary medicines in feedstuffs. Li and co-workers (2009) applied ELISA technology for the determination of the four main nitrofuran parent compounds in feeds. A polyclonal antibody was raised against furazolidone, and this displayed good cross-reactivity towards nitrofurantoin, nitrofurazone and furaltadone. An indirect competitive ELISA based on this antibody was

then developed for the simultaneous determination of four nitrofurans in animal feeds. A limit of detection of 0.2–2.1 μg kg^{-1} was quoted for the method. Sample preparation required solid phase extraction followed by reconstitution either in the mobile phase, for confirmatory HPLC analysis, or PBS for application in ELISA. Recovery ranged from 75% to 87%.

As regards screening assays for coccidiostats, the principal developments have been in the use of biosensors. Biosensors are capable of detecting multiple residues at once with limited sample clean-up required. However, this technology has not been fully exploited in the area of coccidiostats, with only a handful of methods published with single analyte detection. McCarney *et al*. (2003) reported a surface plasmon resonance (SPR) biosensor screening technique for nicarbazin residues. Nicarbazin was extracted from liver and egg using MeCN with liver requiring an additional hexane wash. Development in this area is slowed down by the production and consistent supply of high-quality antibody.

15.7 Future trends

The setting of new limits for the unavoidable cross-contamination of feed batches with coccidiostats in feed and the occurrence of undesirable residues in food from target and non-target animal species should reduce the number of non-compliant residues arising from official inspection in the EU because laboratories will be no longer testing to a zero tolerance. As this legislation has only come into force since mid-2009, a clearer picture will emerge in the forthcoming years. However, it remains a technical challenge for the feed industry to comply with the given maximum cross-contamination rates. However, these questions will be answered through the application of newly developed LC-MS/MS methods, which have only recently been validated to the new criteria and have been implemented in some inspection laboratories. In the area of food analysis several multi-residue LC-MS/MS assays are now in place for detecting residues in eggs and meat. It is important to also highlight that these newly developed LC-MS/MS methods, which allow testing for a broad range of feed contaminants and probably at lower levels than have been tested routinely in the past, have so far been applied mainly in a research environment for the investigation of cross-contamination in discrete studies. Their robustness for routine controls of foods has yet to be demonstrated. In addition, limits have now been set for residues in milk, prompting some groups to commence the development of analytical tests for this matrix. There is little information available at present concerning the excretion levels of feed additive residues in milk following transfer from contaminated feed. In addition, there is no requirement in legislation for monitoring coccidiostat residues in milk samples under 96/23/EC legislation (EU 1996).

Residues of antibiotics in distillers' grains (DG) is a new area of concern for the animal feed sector that has received much media attention recently. Distillers' grains are a major by-product of bioethanol production, which can be used as a major feed or feed ingredient in the cattle, swine and poultry industry. There was a 10-fold increase in DG production in 2008 and it is expected that this trend will increase in future years with an increasing demand for alternative fuel sources (De Alwis and Heller 2010). Antibiotics are added to the fermentation media to increase selectivity by preventing the growth of undesirable bacteria. As a result, there has been concern that residues may be present in animal feed, contributing to the emerge of antimicrobial resistance.

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Antimicrobials in animal feed: benefits and limitations

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Abstract: Considering that most commercial poultry operations house up to 30,000 birds in one house, additives in feed are the most efficient means for delivering treatments, improving health and treating diseases. Typically, antibiotics used in feed are not specifically for therapeutic purposes but rather for growth promotion and improvement of poultry gut health. There is concern that this practice will ultimately result in an increase in antibiotic-resistant bacteria that may be difficult to treat. This chapter discusses the use of antimicrobials in feed, primarily poultry feed, and the benefits of antibiotic usage. The disadvantages, including the development of antibiotic-resistant bacteria, will also be addressed.

Key words: antimicrobial growth promoters, feed medication, poultry, infectious diseases, antimicrobial resistance.

16.1 Introduction: limitations

Antibiotics are the most popular treatment utilized in animal agriculture production around the world. However, risks to human health such as the development of antibiotic-resistant bacteria have become subject to ongoing public debate and regulatory responses. The increasing prevalence of antibiotic-resistant bacteria is of great concern in terms of public health, and the use of antibiotics in animal production has been demonstrated to result in selection for antibiotic-resistant strains of bacteria (Phillips *et al*. 2004). For this reason, countries have posed regulations concerning the use of some antibiotics. However, the US and several of its key trading partners, including Canada and South Korea, differ from the EU in their regulations of the use of antibiotics in animals in two important areas: which antibiotics can be used for growth promotion and the availability of antibiotics to producers. The EU has issued legal provisions that prohibit the use of all

antibiotics in feed for growth-promoting purposes. Moreover, all antibiotics used in food-producing animals require a veterinary prescription (POM – prescription-only medicines). The US, as well as Australia, Canada, Japan, and South Korea, allow the use of some antibiotics for growth promotion (USGAO 2004). New Zealand allows the use of any antibiotics for growth promotion but only those that are not related to antibiotics used in human medicine.

The objective of this chapter will be to review the use of antimicrobials in feed, specifically feed used in poultry rearing. The application(s) of antibiotics in feeds includes treatment of diseases, growth promotion and control of microbial contamination. The link between the use of antibiotics in feed and the development of antibiotic-resistant bacteria will be explored. Finally, antibiotic alternatives that can be used in feeds will be briefly discussed.

16.2 Benefits of antibiotic usage

The history of antibiotics is well known (for review see Jones and Ricke 2003). In 1929, Alexander Fleming discovered penicillin when a small amount of a *Penicillium* fungus contaminated a petri dish with staphylococci. During the 1930s, penicillin was used to treat soldiers with infections or gangrene and could be obtained only from government sources. Penicillin was first used among the US general public in 1942 to treat burn patients with staphylococci infections. Since the mass production of antibiotics began, usage has not been limited to human health alone. Estimates of the amounts of antibiotics used in US animal production range from 3.1 million pounds to approximately 25 million pounds annually (i.e., they range from 13% to 70%; Mellon *et al*. 2001; AHI 2002) and thus vary widely due to the lack of regulations for reporting antibiotic usage. Recent documentation of antibiotic use in chickens has not been done but a more recent report listed general antibiotic use in the US at 50 million pounds in 2008 with 70% being used in the animal industry (Dorit 2009; Muthaiyan *et al*. 2011; Webster 2009).

Antibiotic use falls into two categories in poultry production – therapeutic applications and use as growth-promoting agents at sub-therapeutic levels. The antibiotics used as growth promotants may be the same antibiotics used therapeutically, but preferentially therapeutic antibiotics are not used as growth promotants (Hofacre 2002). Antibiotics can offer producers multiple benefits in addition to improving health and welfare. In particular, an economic advantage can exist in terms of reduced mortality and increased body weight.

16.2.1 Animal health and welfare

In commercial poultry operations, antibiotics are typically used to relieve suffering of sick birds and to minimize the financial impact of disease on

the economic result until the disease source can be identified and eliminated. However, antibiotics may also allow farmers to use less space. Because of the nature of an intensive system, disease can spread rapidly. Without intervention with antibiotics, potentially a whole flock can be lost. For these reasons, without antibiotics, intensive livestock systems might never have become a profitable form of agricultural production (Anson 2009).

Another reason for the use of antibiotics is the potential increase in foodborne diseases when sick birds are sent for slaughtering. For example, Corrier *et al.* (1999) showed that sick poultry that consumed greater amounts of bedding material (litter) had higher rates of *Salmonella* spp. and *Campylobacter* spp. in their intestinal tract. Furthermore, Russell (2003) reported that flocks with higher rates of airsacculitis condemnation exhibited higher levels of *E. coli* and *Campylobacter* spp. in the processed meat.

16.2.2 Growth promotion

The growth promotion effects of antibiotics were first discovered in chickens in 1949. It was demonstrated that chickens fed tetracycline fermentation by-products grew faster as compared to untreated controls (Stokestad *et al.* 1949). Prior to many innovations and advances in poultry science, such as selective breeding and changes in rearing conditions and feed formulation, research indicated a positive growth effect from antibiotics fed at growth-promoting dosages. Early studies, conducted from 1950 to 1960, showed that penicillin could increase mean body weight by 8.5% to 8.8% and by 10.2% to 12.3% when tetracyclines were added to the diet (Heth and Bird 1962). Studies conducted from 1968 to 1980 reported that median body weight increased by 11% when penicillin was used, by 8% to 10% for the tetracyclines, and by 4% to 7% for the 'new' antibiotics (Dafwang *et al.* 1984).

In the 1970s and 1980s numerous articles were published that confirm the beneficial effects of diverse antibiotic substances (flavophospholipol, virginiamycin, zink-bacitracin, tylosin, avoparcin and others) as growth promoters. For more extensive discussions on both the history of growth-promotant antibiotic use and animal agriculture, the reader is referred to the extensive reviews published previously (Bager and Emborg 2001; Jones and Ricke 2003; Butaye *et al.* 2003; Wierup 2001). More recent confirmations of these initial findings come from reports on virginiamycin supplements to a corn–soybean-based diet, which significantly increased weight gain and feed efficiency over the control group but only for the first three weeks of the rearing period (Guo *et al.* 2004). Feeding birds on feed supplemented with lincomycin (2 g/ton starter feed and then 4 g per ton grower feed) significantly increased body weight, feed efficiency and decreased mortality (Sun *et al.* 2005). Chowdhury *et al.* (2009) reported a 10% increase in body weight when a corn–soybean-based diet was supplemented with 0.001% avilamycin. In addition, the incidence of necrotic enteritis increased dramatically after the GPA withdrawal (Tornee 2002). Together, these studies implied that

suppression of infectious disease and stabilization of the gut population can be accomplished with GPA and is important for animal growth. The exact mechanism of how antibiotics increase growth rate is unknown. However, some research has given insight into possible explanations and this research has been reviewed elsewhere (Dibner and Richards 2005).

16.2.3 Economic advantages

The growth period from hatch to harvest for a broiler chicken is very short, six weeks. In that amount of time a broiler chicken will increase its initial weight by 5000 times. Broiler production has changed dramatically from 1955 to the present day. The average market weight of broilers has increased by nearly 50%. Furthermore, the time needed for broilers to reach market weight and the amount of feed required to produce one pound of broiler meat have both declined by approximately 35% (Boyd *et al*. 2001). Industry researchers assert that GPAs are essential to maintaining these increases in productivity and that GPAs have contributed to the decreased cost of chicken products for consumers. One industry estimate reported that a 1.76% increase in poultry production costs would occur from the removal of GPAs, ultimately resulting in an increased cost to consumers of $2.20 per capita per year (NRC 1999).

Few large-scale studies have been conducted to evaluate the cost-effectiveness of GPAs. Furthermore, the available literature is conflicting and seems to be dependent on the antibiotic used. For example, production cost per kilogram of live weight of broilers was lower in chicks fed avilamycin compared to control chicks (Chowdhury *et al*. 2009). Conversely, a large formal economic analysis of GPA use in one large producer conducted over a three-year period reported that feeding a mix including bacitracin, flavomycin, and virginiamycin was not cost-effective and in fact the net effect of using GPAs was a lost value of $0.0093 per chicken (Graham *et al*. 2007). The current studies and literature available are unclear as to any economic value of GPAs in poultry production.

16.2.4 Transmission of zoonotic pathogens

Evidence indicates a direct correlation between animal health and the contamination of the carcass by foodborne pathogens (Singer *et al*. 2007). Diseased animals may shed higher levels of pathogens (e.g. *Salmonella* and *Campylobacter*) than healthy animals (Russell 2003), which may increase the probability of carcass contamination (Olsen *et al*. 2003). In addition, certain animal illnesses may lead to a higher probability of mistakes in the processing plant, including gastrointestinal ruptures (Singer *et al*. 2007). Therefore, antibiotic usage that prevents disease in poultry also can reduce foodborne illness in consumers. There are economic advantages to be considered as well. The estimated burden of foodborne illness due to

Salmonella alone is estimated by the USDA at 2.6 billion dollars annually due to costs associated with loss of productivity, healthcare expenses or premature death (USDA ERS 2007). Thus, it could be argued that antibiotic administration in animal production has an economic advantage by reducing foodborne illness costs.

In short, any economic and growth-promoting advantages attributed to the inclusion of antibiotics in feed remains somewhat contentious at the present time. However, the ideal purpose of antibiotics is for increased health and welfare of the animals and this point is not arguable. Antibiotics, without a doubt, can reduce disease and in turn prevent a reduction in flocks and economic losses. The usage of antibiotics to prevent disease will be addressed in the next section.

16.3 Antimicrobials in feed to prevent diseases

Typically, therapeutic antibiotics are not used as growth promotants (Hofacre 2002). However, the literature is vague as to the effectiveness of growth-promotant levels of antibiotics against colonizing pathogens, largely due to the fact that the mechanisms of growth promotion are unclear. Consequently, there is some potential overlap in terms of bird responses which warrants including the respective antibiotics in any discussion on antimicrobials in feed. Likewise some growth-promotant antibiotics may actually be specifically effective against clinical pathogens and need to be discussed in those terms. Regardless of whether it is being administered for growth-promotant or clinical treatment purposes, the logistics of delivering the proper dosage of antibiotics to every bird within a flock of 30,000 can be challenging and time consuming. Given these scale-up problems associated with delivery, feed and water are the two most efficient routes of application. In most instances, sick birds are treated by delivery of antibiotics in water as sick birds stop eating but will continue to drink water. However, growth-promotant antibiotics are administered only in the birds' feed (Jones and Ricke 2003).

Because sick birds are typically treated by delivery of antimicrobials in water and the focus of this chapter is to discuss antimicrobials in feed, the ability to prevent rather than treat these diseases by administration of antimicrobials in feeds will be the focus of the next section. Out of the multiple diseases afflicting poultry the four most common infections will be discussed in the following sections.

16.3.1 *E. coli*

Escherichia coli infection in poultry is the leading cause of economic loss due to disease for the industry throughout the world (Barnes 2003). It is for this reason that *E. coli* is the primary bacterial infection for which

therapeutic antibiotics are used in treating poultry. *E. coli* (colibacilliosis) infections cause increased mortality and decreased performance in terms of body weight gain (Barnes 2003). *E. coli* is a normal inhabitant of the intestinal tract but imbalances can result in proliferation of *E. coli*. In most instances, *E. coli* infections are secondary infections occurring as a result of a viral infection. Prevention of *E. coli* infections can be achieved with good hygiene and biosecurity practices, including proper ventilation and rodent control programs.

Antibiotics used as growth promotants are generally more effective against Gram-positive bacteria and are poorly absorbed by the gut. For these reasons, GPAs are usually effective only against Gram-positive populations in the gut. However, some literature does indicate that specific antibiotics utilized at growth-promoting levels can be effective against Gram-negatives. Baurhoo *et al*. (2009) demonstrated that growth-promotant levels of bacitracin reduced cecal populations of *E. coli*. Likewise, bambermycin and virginiamycin were both effective at suppressing *E. coli* infection in challenged poults (Fairchild *et al*. 1999). In these studies, no data were collected to further understand the mechanisms of reduction of the *E. coli* populations. Still, it can be inferred that the antibiotics most likely had a positive effect on increasing overall health of the animal which led to a decrease in *E. coli* infection.

16.3.2 Necrotic enteritis

The causative agent of necrotic enteritis is *Clostridium perfringens* which is a ubiquitous bacterium readily found in soil, dust, feces, feed, and used poultry litter. The mechanisms of disease and gut ecology have been reviewed (Cooper and Songer 2009). *C. perfringens* is also a normal inhabitant of the intestines of healthy chickens. However, excessive growth and toxin production results in damage to the intestinal mucosa. In general, conditions that promote the growth of *C. perfringens* or slow feed passage rate in the small intestine could promote the occurrence of necrotic enteritis. Most often the only sign of necrotic enteritis in a flock is a sudden increase in mortality with necropsies revealing gross lesions primarily found in the small intestine (jejunum), which may be ballooned and contain a foul-smelling, brown fluid. Depending on the severity of disease and corrective actions, the disease can persist in the flock for 5–10 days and mortality can be anywhere from 2% to 50% (Anon. 2009).

Because *C. perfringens* is typically present in the intestines, preventing changes in the intestinal microflora is critical to preventing its growth. Therefore, avoiding changes in diet will promote a stabile intestinal microflora and reduce the risk of necrotic enteritis. The addition of ionophores to prevent coccidial damage can be helpful in preventing necrotic enteritis.

Since GPAa are effective against Gram-positive bacteria, *C. perfringens* can be controlled with GPAs. The GPA antibiotics avoparicin, bacitracin,

tylosin, avilamycin, flavophospholipol and virginiamycin have all been shown to be effective at reducing *C. perfringens* and thus necrotic enteritis (Van Immerseel *et al*. 2004). Kaldhusdal and Løvland (2000) reported an epidemic of *C. perfrigens*-associated necrotic enteritis occurred after banning GPAs became effective. Van Immerseel *et al*. (2004) later reviewed the problem of necrotic enteritis after the withdrawal of GPAs. It was pointed out that the incidence of *C. perfringens*-associated necrotic enteritis in poultry has increased in countries that no longer use antibiotic growth promoters and that this may also represent a public health threat as *C. perfringens* can cause foodborne illness.

16.3.3 Salmonella

Early literature does indicate some reduction in cecal populations of *Salmonella* by GPAs. Humbert *et al*. (1991) reported a reduction in *S.* Typhimurium when chicks were fed bacitracin or virginiamycin, but an increase in ST cecal populations in chicks fed avoparcin. George *et al*. (1982) reported chicks fed bambermycin had no reduction in *Salmonella* cecal populations. Conversely, Bolder *et al*. (1999) reported a reduction in *Salmonella* cecal populations in chicks fed bambermycin.

Like *E. coli*, salmonellae are Gram-negative and thus, GPAs typically do not have a direct antimicrobial effect on reducing cecal populations of *Salmonella*, but reductions in cecal populations by GPAs have been reported. George *et al*. (1982) hypothesized the reduction in *Salmonella* was an indirect effect of the GPA that facilitated the production of lactic acid by lactobacilli and may have enhanced the production of volatile fatty acids (VFA) by other microbial species, resulting in an antagonistic effect on *Salmonella*. In support of this, the group showed no inhibition or change in minimum inhibitory concentration over a four-week testing period within a pure culture of *Salmonella*. Thus, no direct antimicrobial effect of bambermycin on *Salmonella* was observed.

16.3.4 Mycoplasma

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are infectious bacteria that cause chronic respiratory disease in chickens. Because mycoplasmas infect the respiratory tract, typical symptoms include coughing, nasal discharge, and air sac lesions, but in some infections no clinical symptoms appear (Feberwee *et al*. 2005). The infectious dose is very low and chickens may be inoculated with as few as 100 cells. Infected animals can excrete the bacteria as soon as three days post-inoculation (Feberwee *et al*. 2005). Both horizontal and vertical transmission has been shown to occur rapidly (Soeripto *et al*. 1989).

The two most common *Mycoplasma* species, *M. gallisepticum* and *M. synoviae*, may significantly impact breeder, broiler, and egg laying

production. Reductions in egg production are estimated at $140 million annually (Peebles *et al*. 2006). In broilers, a reduced feed conversion efficiency, depressed growth rate, and condemnation of carcasses have led to losses as high as $750,000 from a single outbreak of MG (Evans *et al*. 2005).

Mycoplasma infections are treatable with antibiotics, and drugs commonly applied include chlortetracycline, tylosin, and tiamulin. However, treatment may not be fully effective at clearing the infection (Gautier-Bouchardon *et al*. 2002; Reinhardt *et al*. 2005). Therefore, prophylactic treatment with antimicrobials delivered via feed has been investigated due to the devastating economic impact of *Mycoplasma* infections. Tiamulin, an antibiotic of the pleuromutilin group, has been investigated for effectiveness against *Mycoplasma*. Although the drug is effective, unfortunate side-effects including reduced growth performance and high cost make feasibility difficult for most poultry operations (Islam *et al*. 2008). Currently, depopulation of sick birds is still the most common approach for the containment of *Mycoplasm*a infections.

16.4 Development of antimicrobial-resistant bacteria

It is generally argued that the increased resistance of bacteria to antibiotics is due in part to the administration of antibiotics for both growth-promotant and veterinary purposes (Phillips *et al*. 2004). A reduction in the choices of antibiotics to treat pathogenic bacteria due to the development of antibiotic resistance is not the only concern. Commensal bacteria may also develop resistance genes and harbor these genes for extended periods of time. Even after antibiotic usage has ceased, commensal bacteria may still have the ability to transfer the resistance genes to pathogens. Therefore, a major question surrounding antibiotic resistant bacteria research is how long does it take after antibiotics are no longer used before antibiotic resistance is decreased?

Increased human health risks associated with antibiotic resistance are the primary driving force associated with the use of antibiotics in animal husbandry (Singer and Hofacre 2006). Poultry can be a source of several foodborne pathogens, including *Salmonella* and *Campylobacter*, and the literature is clear that drug-resistant strains have been on the rise. However, without proper baseline studies, it is difficult to correlate GPAs with antibiotic resistance and, furthermore, it is difficult to predict if the removal of GPAs from production will have a positive impact by reducing the resistance of bacteria to antibiotics.

For some insight, researchers are turning to organic poultry production systems because, by definition, these birds are raised without any antibiotics. A majority of the reports indicate that the prevalence of *Salmonella* is increased within organic systems and antibiotic-resistant isolates are being recovered despite the fact that antibiotics are not used (Bailey and Cosby

2005). Therefore, continuing research will most likely be needed to understand differences in antibiotic resistance and rearing conditions.

16.4.1 *Salmonella*

In the US, it is difficult to correlate antibiotic usage in feed with human salmonellosis. This is due in part to the lack of regulations in the US that require reporting of the use of some antibiotics in production systems (Graham *et al*. 2007). However, it appears that the incidence of salmonellosis in the US has remained unchanged over a period of two decades while production practices have been modified over the same time frame. Likewise, antibiotic resistance can still be found even in *Salmonella* serotypes isolated from poultry farms where antibiotics are not traditionally used (Melendez *et al*. 2010). The Danish experience with human salmonellosis and GPAs has been different. After the GPA ban, microbiologically confirmed infections of salmonellosis increased in prevalence in Denmark after they had declined for three years (MFAF 2001). Furthermore, an increase in resistance of tetracycline and sulphonamides in *S.* Typhimurium isolates from pigs and from human domestic infections occurred and was speculated to be in response to the increased use of therapeutic antibiotics in Denmark in 2001 (DANMAP 2001).

Antibiotic resistance in foodborne *Salmonella* isolates could be a public health concern, since *Salmonella* is a leading cause of foodborne illness around the world with poultry and poultry products being linked to a majority of the cases of foodborne salmonellosis (USDA ERS 2007). Most serovars of *Salmonella* may colonize the gut of poultry without causing disease or other adverse effects to the health of the animal. During processing, if intestinal rupture occurs, contamination of the carcass can occur and consuming undercooked or raw contaminated poultry can lead to infection with *Salmonella*. Similarly, eggs can be a source of salmonellosis. In laying hens, the reproductive tract or the intestinal tract may be colonized and eggs can become contaminated by horizontal (reproductive tract) or vertical (fecal contamination) transfer (Dunkley *et al*. 2009).

The incidence of non-typhoid disease in the US has been stable since 2004 but has decreased by approximately 8% when compared to the prevalence data collected from 1996 to 1998 (CDC 2007). Non-typhoidal *Salmonella* prevalence is reported at 14.9 cases per 100,000 of laboratory-confirmed cases annually. However, the true prevalence of non-typhoid *Salmonella* in the US is thought to be under-reported, with 38 cases going unreported for each culture confirmed case, which would result in 520 cases per 100,000 (Voetsch *et al*. 2004; Weinberger *et al*. 2005).

16.4.2 *Campylobacter*

Campylobacter, like *Salmonella*, is a leading cause of foodborne illness and also can be carried in the gut of poultry as a commensal. *Campylobacter* is

also Gram-negative and therefore the literature indicates most GPAs do not have a direct antimicrobial effect, but that GPAs can be effective at reducing cecal populations (Baurhoo *et al*. 2009). Any effect of GPAs on *Campylobacter* is most likely explained by the same mechanism that affects *Salmonella* (see Section 16.3.3). *Campylobacter* is highly susceptible to low pH and, for this reason, increases in lactic acid concentration produced by *Lactobacillus* populations can limit colonization with *Campylobacter* (Lee and Newell 2006).

Campylobacter resistance to fluoroquinolones is of great concern, so much so that from 12 September 2005, the FDA withdrew approval for the use of fluoroquinolones in poultry production (FDA 2005) due in part to multiple studies finding links between antibiotic resistance of *Campylobacter* and fluoroquinolone use in live poultry (Luo *et al*. 2003; Alfredson and Korolik 2007). Ciprofloxacin (a popular fluoroquinolone) is one drug of choice for the treatment of campylobacteriosis (Alfredson and Korolik 2007) because *Salmonella* and *Shigella* infections have symptoms that are very similar to campylobacteriosis and these organisms also are easily treated with ciprofloxacin in human patients. This makes treatment for gastroenteritis with ciprofloxacin advantageous because no fecal culture results are needed prior to initiation of treatment. Unfortunately, increasing incidences of ciprofloxacin-resistant *Campylobacte*r may reduce or eliminate this treatment option.

The emergence, mechanisms of development, and persistence of *Campylobacter* antibiotic resistance have been reviewed recently (Luangtongkum *et al*. 2009). The review points out literature that demonstrates the persistence of fluoroquinolone resistance despite the cessation of use of the drug both in live animals that are colonized and from carcasses that were purchased at retail (Luo *et al*. 2004; Nannapaneni *et al*. 2009). Studies done in France are encouraging and show that ciprofloxacin-resistant *Campylobacter jejuni* isolated from broiler carcasses decreased from 16.8% in 1999 to 9.4% in 2004 (Gallay *et al*. 2007), suggesting that the limiting of fluoroquinolone use in poultry reduced antibiotic resistance in *Campylobacter*. Future monitoring is needed to determine if levels of ciprofloxacin-resistant *Campylobacter* levels from poultry flocks and products will continue to occur to give physicians information that they will need to treat patients effectively.

16.4.3 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is a pathogen that is typically associated with serious community-acquired and nosocomial disease (Kennedy *et al*. 2008). It is the second most common bacterium isolated from blood cultures and is by far the most common hospital-acquired infection (Anon. 1997). Of greatest concern are strains of *S. aureus* with resistance to methicillin (MRSA). Although most *S. aureus* infections are hospital acquired, transmission of

MRSA from pigs to pig farmers and their families has been documented (Voss *et al*. 2005), but these transmissions concern ST398, the so-called livestock-acquired MRSA, which in contrast to the hospital-acquired MRSA (HA-MRSA) has a very low pathogenicity and is hardly transmitted horizontally (between humans). Furthermore, the isolation of MRSA from raw beef, pork and poultry meat has been reported (Lee and Newell 2006; Pereira *et al*. 2009; Pu *et al*. 2009).

The issue of MRSA in food animals is an emerging concern. Studies have been published in which MRSA isolates obtained from meats were typed and compared with genotype databases. In this way, researchers have been able to determine that these strains in meat and meat products were in most cases of human origin and not zoonotic genotypes (livestock acquired). This means that MRSA is present in the food chain but most likely due to human contamination (Pu *et al*. 2009). For this reason, attention needs to be focused on preventing the introduction of MRSA from human carriers onto the meats they handle which can result in spreading of the pathogen.

16.4.4 *E. coli*

As discussed earlier, *E. coli* is the leading bacterial infection in poultry which therapeutic antibiotics are used to treat. It has been speculated that a limited choice of antibiotics to treat *E. coli* infections in poultry created selection pressure that drove the emergence of multi-drug-resistant *E. coli* (Singer and Hofacre 2006). Some of the first research investigating any linkage among antibiotic usage in poultry, the development of antibiotic-resistant bacteria and human clinical isolates was focused on *E. coli*. A study conducted from 1957 to 1960 reported an increase in the prevalence of tetracycline-resistant *E. coli* (Sojka *et al*. 1961) which also correlated with an increase in feeding tetracycline to broilers. These findings resulted in a general ban on tetracyclines (fermentation cake) as antimicrobial growth promoters. Further research in that decade reported an increase in pathogenic *E. coli* being isolated from humans and animals (Smith 1966).

The burden of human disease due to *E. coli* infections is enormous with an estimated incidence of 150 cases per 100,000 people per year (Jackson *et al*. 2005). The increase in multi-drug-resistant *E. coli* compromises and even eliminates treatment options. Exploration of origins and sources needs to be a priority to facilitate control of these bacterial strains. Animal origins have been investigated as a source of an outbreak of some drug-resistant strains of *E. coli*. In this investigation, a high prevalence of *E. coli* urinary tract infections (UTI) over multiple states was investigated as it was also noticed that multiple people in the same area had intestinal colonization with the same *E. coli*. This led the investigators to believe that food animals were the source of the *E. coli*. The investigation was not able to link the UTI outbreak to animal origins; however, the authors pointed out that a limited number of animal samples may have limited the ability to pinpoint

the source of the *E. coli* and further sampling was required (Ramchandani *et al*. 2005).

Tetracycline and sulfonamide resistance in human clinical *E. coli* isolates continues to be reported by many diagnostic laboratories (Zhao *et al*. 2005; Singer and Hofacre 2006). In addition, ciprofloxacin-resistant *E. coli* is being isolated from humans and poultry (Thorsteinsdottir *et al*. 2010). The development of ciprofloxacin in *E. coli* is quite alarming due to the fact that *E. coli* infections are prevalent and ciprofloxacin is a last line of defense if other drugs are ineffective (Johnson *et al*. 2005). Recent literature indicates that ciprofloxacin-resistant *E. coli* has emerged in Iceland. This is an interesting development primarily because antibiotics are not used in poultry operations in that country, and houses are cleaned and disinfected between flocks (Thorsteinsdottir *et al*. 2009). For these reasons, a persistent environmental source is unlikely. Recent published reports on this problem found that feed was implicated as a source of the *E. coli* (Thorsteinsdottir *et al*. 2010). The study isolated ciprofloxacin-resistant *E. coli* from feed, broilers and humans. However, the genotyping of the drug-resistant isolates did not conclusively link the isolates from poultry and poultry feed with human *E. coli* strains.

The lack of conclusive proof linking animals as sources of drug-resistant *E. coli* isolates from humans should not allow researchers to turn their focus away from this issue. However, the disease burden of *E. coli* infections and the emergence of multi-drug resistance warrants constant monitoring of these strains. However, as discussed, the emergence of multi-drug resistance is not only a problem with *E. coli*, but also a problem with multiple pathogens, which means an aggressive monitoring program is needed to pinpoint sources and design corrective actions.

16.5 Antibiotics and poultry gut health

The function of an immune system is to consistently defend the body from potential detriment caused by invading microorganisms. Although the GI tract contains a vast array of microbes, the majority of these microbes are beneficial to the host. The bacteria present in the gut can provide multiple benefits, including the synthesis of vitamins and educating the immune system (Xu and Gordon 2003). For this reason, the microbes in the gut can have profound effects on gut health. Since antibiotics can affect the microbes present in the gut, the antibiotics as well can have an impact on poultry gut health. These issues will be discussed in the following section.

16.5.1 The immune system

Prior to birth, neonates have a gastrointestinal tract (GIT) that is free of microbes. The initial colonization of the GIT in part defines what the

immune system will consider as commensal or pathogenic. Therefore, considerable amounts of research have been done concerning the administration of probiotics to newly hatched chicks, the hypothesis being that if chicks are colonized rapidly and the immune system is only aware of commensal bacteria, than a more aggressive immune response can be elicited towards pathogens.

As stated, the beneficial microbes play an important role in educating the immune system as to which organisms can be tolerated and those that should be eliminated by the immune system (Xu and Gordon 2003). In fact, it has been demonstrated that germ-free animals have an inadequate immune system compared to their colonized counterparts (Berg 1983). The immune system of germ-free animals is thought to be 'under educated' and for this reason an acute systemic response is initiated when faced with any bacteria in the gut. An acute immune response can result in a slower growth rate of the bird because nutrients are redirected that are needed for immune response instead of being used for growth-related processes including skeletal muscle growth (Ferket and Qureshi 1992).

Antibiotics can facilitate the education of the immune system. The administration of antibiotics in feed can reduce pathogenic bacteria and allow the commensal population to colonize more efficiently. Furthermore, pathogenic bacteria that colonize young poultry can cause inflammation and diarrhea which will flush the gut of not only pathogenic but also commensal microbes, which can be detrimental to the gut bacterial population.

16.5.2 Gut histology

Gut histology, like the immune system, can be profoundly influenced by the bacteria present in the gut. For example, microbial products such as ammonia (NH_3) and lactic acid are produced by commensal bacteria including *Lactobacillus*. These products can increase enterocyte (absorption cells) cell division and alter mucosal barriers (Zimber and Visek 1972). These alterations to the gut histology can result in a longer intestinal tract.

Since antibiotics can reduce bacterial populations in the gut, the microbial by-products produced by these organisms are also reduced. For this reason, animals fed antibiotics are reported to have a reduced weight and length of the intestines compared to birds not fed antibiotics (Postma *et al.* 1999). This reduced intestinal profile is similar to that observed in germ-free birds. In fact, Stutz *et al.* (1983) reported reduced lamina propria, lymphoid tissue, reticuloendothelial cells, intestinal weight and moisture in germ-free birds, which demonstrates the large effect gut microbes can have on the histology of the gut.

16.5.3 Gut ecology

Due to the importance of the commensal microbes early in the life of the bird, it is critical to maintain and stabilize the populations in the gut as

rapidly as possible. Since antibiotics can ward off colonizing pathogens, the gut populations can be established in a timely manner. The stabilization of the gut flora also has a consistent protective effect later in life as well as early in life. For example, birds that are given antibiotics are protected from necrotic enteritis and *E. coli* infections due to this stabilized gut population (Dahiya *et al*. 2006).

The regular use of antibiotics in feed can have an extensive effect on the gut health of poultry. There is no doubt that decreasing the risk of pathogenic infection in poultry is a benefit of antibiotics. Some argue that antibiotics act in a way that weakens the immune system due to the effects on gut histology as described in the previous section. However, this point can be countered with the position that the life cycle of a broiler from hatch to harvest is a short 35 days and if the immune system is slightly underdeveloped it is of no consequence due to the short grow-out time. For other poultry, such as laying or breeding stock, these immune system problems may be an issue because these birds have a life span of one to two years. Much of this may be dependent on how the administered antibiotic interacts with the gut microflora and the timing of that interaction. It has become quite clear with more advanced molecular approaches that early establishment of microflora such as probiotic cultures can lead to early development the immune system in poultry (Farnell *et al*. 2006; Yoshimura *et al*. 2010). Biological agents that disrupt this interaction may open the door for pathogen establishment before the gut microflora can become stabilized.

16.6 Future trends

The primary concern of consumers concerning the use of antibiotic usage in animal production is increased resistance of bacteria to antibiotic treatments. For this reason, much research has focused on alternative treatments to antibiotics both for growth promotion and for antimicrobial effects. Although alternatives to antibiotics such as probiotics are gaining popularity, problems such as effectiveness and delivery have prevented probiotics from becoming more widely used. The use of alternatives to antibiotics as additives in feed has been extensively reviewed (Ricke *et al*. 2005; Maciorowski *et al*. 2006; Windisch *et al*. 2007). Ricke *et al*. (2005) reviewed the addition of chemicals to or the physical treatments of feed to reduce foodborne pathogens. Van Immerseel *et al*. (2006) reviewed the use of organic acids in feed to reduce *Salmonella* in poultry feed and intestinal colonization. The use of phytogenic products (plant extracts) in feed has also been reviewed (Windisch *et al*. 2007). For more details see also Chapter 17.

Common problems encountered with alternatives include delivery and costs. For example, the use of bacteriophages is a promising alternative because specific organisms can be targeted and eliminated (Joerger 2003;

Maciorowski *et al*. 2007). However, delivery can be complicated because bacteriophages are viable and maintaining viability can be challenging. Bacteriocins are another alternative that has been promising but the cost associated with production of mass quantities currently eliminates them as a practical option (Joerger 2003). Clearly, more work needs to be done in adapting alternative options for use in large-scale poultry production.

Moreover, from the available studies, it is clear that removal of antibiotics does not always result in an immediate reduction of antibiotic-resistant bacteria. Antibiotic resistance is thought to serve as a competitive advantage to bacteria, but only in the presence of antibiotics. It is generally believed that the acquisition of drug resistance, especially chromosomal mutations, involves a fitness cost to the bacteria in the absence of the selective pressure produced by antibiotics (Andersson and Levin 1999; Levin *et al*. 2000). But this is not always the case and, in fact, some antibiotic-resistant bacterial strains have been shown to be 'more fit' and to outcompete their non-resistant counterparts in animal colonization studies (Luo *et al*. 2004). For these reasons, future monitoring of antibiotic resistance is necessary.

A consistent monitoring program will serve multiple purposes. Physicians will be aware of the antibiotics that may or may not be effective against bacteria. Secondly, surveys of bacteria collected from feed and other farm environments can locate sources. These sources can then be controlled, thus controlling the dissemination of antibiotic resistance further down the food chain. Finally, corrective actions can be validated with monitoring and a decision can be made whether the corrective actions were effective or not.

16.7 Acknowledgments

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16.8 References and further reading

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17

Alternatives to antimicrobial growth promoters (AGPs) in animal feed[1]

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Abstract: Antimicrobial growth promoters (AGPs) are antibiotics used to both protect animal health and stimulate growth. The ban on their use means there is an urgent need to find more acceptable alternatives. This chapter reviews research into the range of alternatives such as veterinary homeopathy, isotherapy, phytotherapy and the use of novel feedstuffs.

Key words: antibiotics, antimicrobial growth promoters, animal farming, alternative feed additives.

17.1 Introduction

The ban on antimicrobial growth promoters (AGPs) has given rise to a number of practical problems in animal husbandry in Europe. These antibiotics were used to protect the health of the animals but also had a positive effect on their growth rate, as well as on feed efficiency. The ban on these molecules has therefore resulted in a reduction in animal performance and thus also in economic losses. In addition, the withdrawal of AGPs makes the animals more sensitive to infections and they may consequently develop severe diseases that require veterinary care, and that can cause reduced weight gain and even mortality in some cases. All these deleterious effects have a significant economic impact (Kjeldsen, 2005). Hence, finding alternatives to AGPs is of crucial importance for European farming.

[1] In Europe the abbreviation AGP is common, whereas in the US and other continents, the same group of substances is described as growth promoting agents (GPAs).

17.2 Chronology of the ban on antimicrobial growth promoters (AGPs) in Europe

Antibiotic growth promotion in agricultural animal production, particularly for monogastric animals, has been practised for about 50 years as reviewed by Dibner and Richards (2005).

Antibiotic growth promoters have been in the process of being phased out in the European Union (EU) for some time, but a complete ban on their use was only implemented in July 2003. The use of the remaining four growth promoters was gradually reduced until, in January 2006, the ban became fully effective.

17.3 Main consequences of the ban on antimicrobial growth promoters (AGPs) in Europe

As mentioned above, animals whose diet does not contain AGPs often become more sensitive to infections and in consequence may develop severe diseases requiring veterinary care. In many countries this resulted in an increase in the use of therapeutic antibiotics after AGPs were banned. This increased use of the same or similar molecules could actually increase the likelihood of the emergence of resistant bacteria, which is a source of increasing public health concern, especially when zoonotic pathogens are involved.

Dibner and Richards (2005) provide an excellent overview of the consequences of the AGP ban in Denmark, indicating that the significant reduction (54%) in the total use of antibiotics in food-producing animals was accompanied only by an increase of 5% in the use of antibiotics licensed for therapeutic use. An undesirable side-effect of the AGP ban was an increase of the prevalence of necrotic enteritis in poultry, which was counteracted by a significant increase in the use of the ionophoric coccidiostat salinomycin, which is known to be active also against *Clostridium perfringens*.

In addition, Kjeldsen (2005) demonstrated that in Denmark, the use of therapeutic antimicrobials in pig production increased after the ban on AGPs was put into place, due to increasing problems with diarrhoea in weaned animals when AGPs ceased to be used. At the same time, average daily weight gain in pigs decreased and mortality rates increased from 2.7 to 3.5% (Callesen, 2003; Dibner and Richards, 2005). This data will be an interesting tool in future years, as it will allow the effect of the ban on AGP to be assessed (Dibner and Richards, 2005). The observations made in Denmark could also prove to hold true in other European countries, i.e. the AGP ban would be followed by an overall increase in total antibiotic consumption, presumably as a result of the greater need to treat clinical disease outbreaks which would previously have been suppressed through the use of AGPs.

17.4 Mode of action of antimicrobial growth promoters (AGPs) in animal production and possible alternatives

The characteristics of AGPs help to explain their previous position as the additives of choice for growth promotion. In general they are effective at remarkably low doses and are relatively cheap, thus yielding significant return on investment. The search for replacements has been severely hampered by a lack of understanding of how AGPs work. It is widely assumed that AGPs act mainly through their effect on intestinal microflora. With less than 10% of intestinal microflora so far identified, there has been little chance to fully explore the specific effects of AGPs. It is postulated that AGPs benefit livestock by reducing the total number of intestinal microorganisms, including pathogenic microorganisms, and/or by creating a more favourable balance between beneficial and non-beneficial microorganisms. The intestinal microflora have important and differing effects on animals, including regulation of epithelial cell turnover, competition for ingested nutrients, modification of digestion, competitive exclusion of pathogens, metabolism of mucus secretions and modulation of mucosal immunity. The best attempts at explaining the mode of action of AGPs are summarised in the study by Bikker and van der Aar (2005). However, responses to AGPs are subject to significant variation and may, to a large extent, be dependent upon the environment in which the animals are raised and the diet offered to them.

AGPs have proved to be an effective method of enhancing animal health, uniformity and production efficiency. Their removal has had a number of consequences, and will therefore be a difficult obstacle to overcome, particularly if European animal production is to remain competitive with that of the rest of the world, where such products are likely to remain in use. In addition to this, regulation regarding zoonotic diseases (EC 2160/2003b) requires that member states better control, or even eradicate, zoonotic pathogens. The combination of these factors has led to various new research activities with the following objectives: (1) pathogen reduction, (2) augmentation of the immune response of the animals, and (3) development of nutritional strategies and/or use of feed additives that either improve performance in their own right, or directly modify the microbial flora of the gut.

Aspects of pathogen reduction have already been discussed in this book and thus will not be further developed in this chapter. We would just like to mention that since the ban on AGPs, new management practices and strategies developed today all aim to achieve improved hygienic conditions at the level of farms and feed mills.

17.5 Traditional therapeutic approaches as an alternative to antimicrobial growth promoters (AGPs)

Improvement of the animal's inherent immune response is one of the objectives of recent studies in the field (Berghman *et al.*, 2005). Tailored

vaccination programmes combined with the development of improved vaccine delivery may provide opportunities for minimising feed medications. At the same time, knowledge is increasing of how to control the negative effects on performance that the immune response causes. It seems that the systemic, acute-phase response of the animal to disease challenge involves a significant nutrient requirement, meaning that fewer nutrients are available for metabolic use. Conjugated linoleic acid is one compound that has been investigated for its apparent abilities to alleviate the immune-associated anorexic response (Klasing, 1998).

In the meantime, alternative methods are being developed in the field, such as homeopathy, isotherapy and phytotherapy. Homeopathy is a therapy based on the practice of treating like with like. A disease is treated using an agent or substance that produces symptoms in a healthy individual similar to those experienced by a sick individual. Homeopathic medicines contain often very small quantities (high dilutions) of the agent/substance prepared in a special way. In the organic livestock sector, homeopathy is the alternative therapeutic approach commonly used to replace antibiotics. Homeopathy has demonstrated its effectiveness in practice in a range of medical areas, but scientific evidence is lacking. The research literature that looks at veterinary homeopathy consists of fewer than 20 published, peer-reviewed randomised controlled trials (Mathie *et al.*, 2007). The research data available relate to the treatment of mastitis and infertility in cattle, infectious diseases including colibacilloses in pigs, growth rate in pigs and salmonella in chickens (Camerlink *et al.*, 2010). Homeopathic remedies may offer some benefits as no residues remain in the animal products, nor does homeopathy generate resistant microorganisms. Homeopathy aims to activate the self-healing mechanisms of the body. The healing process could therefore take longer, and more attention might need to be paid to determining the correct remedy. A lack of knowledge and understanding of homeopathy could account for its currently limited use in the livestock sector. In addition, research in the field of homeopathy is often subject to criticism. One reason for this is that at the molecular level no actual substance can be detected in some highly diluted homeopathic medicines. Detractors claim that veterinary practitioners who use homeopathic remedies are using their position of authority to convince the owner that the animal being treated by homeopathic methods is getting better (Camerlink *et al.*, 2010).

Isotherapy is the specialised application of homeopathic theory and therapy. An isotherapic remedy is prepared from the blood or other secretion of an infected individual, diluted to the same extent as in regular homeopathic medicine. As with homeopathy, the research literature on isotherapy is scarce. A systematic analysis of selected papers where animal models are used for studying isotherapy showed that methodological rigour is generally adequate, even if some particular aspects could be still improved (Bonamin and Endler, 2010).

Phytotherapy is the treatment of a disease using natural plants or plant extracts. Some involved in animal production claim that these alternative methods give satisfying results in the field for the control of diseases such as coccidiosis; however, in many cases the lack of coherent information about product composition, as well as about the experimental design used in some studies, hinders a scientific evaluation of their efficiency. Indeed, while an abundant bibliography suggests that various plants or plant extracts, either pure or in combination, may improve the animal's health and/or performance, most of t he studies have been performed on mixtures of whole plants or parts of plants (leaves, buds, bark, bulbs, roots), extracts or decoctions of plants where neither the proportion nor the mode of extraction is indicated. It is difficult, then, to disclose the nature of the extracts and the active ingredients that are exerting the reported beneficial effects. In addition, the experimental conditions (animal types, rearing conditions, dietary ingredients, feed formulation, production levels, health status, current diseases, and so on) are not always indicated, are extremely diverse and are often not reflecting European rearing systems. Subsequently, the claimed beneficial effects are not reproducible or the observed effects are very limited, lacking statistical significance (AFSSA, 2007).

17.6 Novel nutritional strategies and feed additives

The link between diet and the incidence of enteric disease in monogastric animals is well known. The following sections describe some nutritional strategies and/or feed additives that either are able to improve performance in their own right, or help to directly modify the gut microbial flora.

17.6.1 Feed formulation and preparation

In the absence of antimicrobial growth promoters, ingredients that potentially increase the risk of adverse health effects must be used with greater caution. For instance, diets based on rye and barley, and to a lesser extent wheat, seem to lead to greater susceptibility to necrotic enteritis (Riddell and Kong, 1992). Indeed, these feedstuffs have long been known to be of poorer nutritive quality than corn, probably because of the presence of large quantities of soluble, viscous arabinoxylans and β-glucans, which significantly reduce the rate of digestion. A reduced rate of digestion causes greater substrate provision for microflora resident in both the lower small intestine and the large intestine/caecum. In turn, a high substrate availability in the lower gastrointestinal tract increases the risk of bacterial overgrowth (the bacterial population has been shown to be 100 to 1000 times larger with rye compared to corn-based diets), including the growth of pathogenic bacteria (Apajalahti and Kettunen, 2003).Gut health and enteric disease resistance are thus often dependent upon the digestibility of feed

components and feed formulation, and any approach that improves digestibility is typically beneficial for the animals.

The cereal base can affect not only total numbers of bacteria in the gut, but also the profile of the gut microflora (review of Burel and Valat, 2009).[2] For instance, when rye is used instead of wheat in the diet of broiler chickens, the relative abundance of the *Streptococcus/Enterococcus* group and *E. coli* increases, whereas the abundance of the *Lactobacillus* group drops dramatically (Apajalahti and Kettunen, 2003), and when wheat and barley are used in the diet instead of corn, facultative anaerobic bacterial populations increase, including those of lactobacilli and coliforms (Mathlouthi *et al*., 2002). According to Apajalahti and Kettunen (2003), it seems that corn selects bacteria other than bifidobacteria. Indeed, corn, as well as sorghum, stimulates enterococci. But the strongest effect seems to be the stimulation of streptococci by rye-based diets. An increase in streptococci, enterococci and coliform populations is likely to cause additional stresses that affect the performance and health of the animals. In other respects, poorly digested protein meals cause the proliferation of putrefying bacteria in the hindgut, which increases toxic metabolites that compromise gut health. In general, antibiotics are most effective in animals fed diets containing high levels of non-digestible proteins (Smulders *et al*., 2000).

The digestive microflora can also be modified by the form in which the diet is fed (whole grains, meals, pellets), the grain type and the particle size (Santos *et al*., 2008; see also the review of Burel and Valat, 2009). Pelleting contributes to an increase in coliforms and enterococci in the ileum, and a reduction of *Clostridium perfringens* and lactobacilli in the distal parts of the digestive tract (Engberg *et al*., 2002). Feeding whole or coarsely ground grains decreased caecal *Salmonella* populations in 42-day-old broiler chickens (Santos *et al*., 2008). The consumption of a whole wheat-based diet compared to a ground wheat-based diet also caused a change in the microflora: a decrease of the ileal population of coliforms and lactobacilli at the beginning of the rearing period of broiler chickens (Gabriel *et al*., 2003). This is extremely significant, as there has recently been an increasing interest in the use of whole grain in the feed of poultry in order to decrease feed cost and also to meet consumer demands for a more 'natural' feeding system and improved animal welfare. But more generally, it seems that feed processing significantly affects the characteristics of the feed as a substrate for the bacterial community. The temperature of the conditioning process, the pressure of the steam and so on may all add to the characteristic structure of the bacterial community. Thus, the manufacturing process itself could be used to control and manage the GI microflora of animals (Apajalahti and Kettunen, 2003).

[2] Parts of Section 17.6.1 are based on Burel C. and Valat C. (2009). Material has been reproduced with permission from Wageningen Academic Publishers.

The immune system of an animal is also affected by nutrition, as discussed in detail in the works of Klasing (2007), Kogut (2009), Kogut and Klasing (2009) and Bao and Choct (2010). The commensal microflora play a role in competitively excluding pathogens, thus providing significant benefits to the intestinal immune system. The composition of the diet can provide the basis for the establishment of the commensal microflora and thus also the development of the intestinal immune system in young animals. However, some cereals contain high proportions of non-starch polysaccharides (NSPs) and oligosaccharides, the effect of which on the immune system of animals has not yet been definitively established. Choct (1997) reported that the amount of fermentative microflora in the small intestine of chickens is significantly reduced by the depolymerisation of soluble NSPs. Given that nutritionists are increasingly forced to introduce plant-based by-products in their diet formulations for farm animals, it has become even more important for researchers and animal producers to gain an understanding of the mechanisms through which NSP and its associate substrates influence the intestinal immune system, and thus the overall health of the animals (Bao and Choct, 2010). It seems that NSPs might not only provide substrates for beneficial bacteria but could play an important role in removing free radicals, and act as antioxidants.

Given that the criteria used to formulate animal feeds do not usually take into account the importance of nutrition in maintaining animal health, the modification of nutrition for farm animals to improve their intestinal health has an important role to play in modern agriculture.

17.6.2 In-feed enzymes

Feed enzymes also play an important role as feed additives. Typical examples are carbohydrases (α- and β-amylases, cellulose, α-galactosidase, β-glucanase, β-glucosidase, glucoamylase, hemicellulase, invertase, β-mannanase, lactase, pectinase, pullulanase, xylanase), proteases (protease, bromelain, ficin, papain, pepsin, trypsin), lipase, oxidoreductase (catalase, glucose oxydase) and phosphatase (phytase). These enzymes can increase the digestibility of nutrients, decrease intestinal viscosity and inactivate antinutritional factors in monogastric animals, leading to greater feed efficiency and performance. At the same time they can help to minimise the negative environmental impact of increased animal production. However, the relative contribution of an enzyme preparation is greater when the quality of the feedstuff is lower. For instance, the digestibility of wheat, barley, rye, triticale and even corn-based diets can be significantly improved through the use of exogenous enzymes including xylanases, phytases and -glucanases (Rosen, 2001). In addition, young animals such as newly weaned piglets may lack the required amounts of certain enzymes to digest feed ingredients. Therefore, adding enzymes to their diet may be a useful strategy to increase feed digestibility,

improve early growth performance and limit the occurrence of diarrhoea in recently weaned pigs.

Because supplemental enzymes mediate their beneficial effects primarily by enhancing feed digestibility and nutrient availability to the host, they also influence the microbial ecosystem of the gut (Choct *et al*., 1996; Hock *et al*., 1997; Bedford, 2000; Rosen, 2001; Parker *et al*., 2007). When fermentable substrates are removed from the ileum, ileal populations of microflora are reduced (Bedford and Schulze, 1998). While the addition of exogenous enzymes appears to limit microbial growth in the ileum, the opposite may be true in the caecal environment. Here, the products of enzymatic breakdown may provide fermentable substrates to the caecal flora. Increases in volatile fatty acid (VFA) production and changes in the VFA profile favour the beneficial organisms (*Bifidobacteria*, for example) and suppress populations of deleterious organisms (*Campylobacter, Salmonella, Clostridium*). The use of these enzymes as feed additives is restricted in most countries pending approval by local authorities, or within the EU through authorisation by EFSA's FEEDAP (Feed and Feed Additives Panel) in accordance with Council Regulation 1831/2003 (EC, 2003a).

17.6.3 Prebiotics

When specific carbohydrates are included in the diet, preferably fermented by beneficial microorganisms, they not only modify the availability of nutrients from raw ingredients for bacterial fermentation, but also have an osmotic effect and can positively influence the composition of the GI microflora. Prebiotics are defined as 'non-digestible or low-digestible food ingredients that benefit the host organism by selectively stimulating the growth or activity of one or a limited number of beneficial bacteria in the distal part of the GI tract' (Crittenden and Playne, 1996). Indeed, specific species can be selected for prebiotics which escape digestion by the host, but are readily available to the metabolic machinery of the target microorganisms (bifidobacteria and some Gram-positive bacteria).

Prebiotics are short-chain carbohydrates that cannot be digested or absorbed by the animals and are therefore available to the intestinal microflora. They are also known as 'dietary fibres'. The ileal digestibility of inulin (a prebiotic) is only 7.5%, but none can be found in faeces, indicating total fermentation in the hindgut. The end products of fermentation are short-chain fatty acids. There are two main types of prebiotics: the fructo-oligosaccharides (FOS) and the mannan-oligosaccharides (MOS). Briefly, the FOS have been shown to influence the intestinal bacterial population by enhancing the growth of lactic acid bacteria (*Lactobacillus* species and *Bifidobacterium*) and to inhibit *Escherichia coli* and *Salmonella* growth in the large intestine (Hidaka *et al*., 1986; Mitsuoka *et al*., 1987; Roberfroid *et al*., 1998; Fukata, 1999; Xu *et al*., 2002). Beneficial bacteria such as *Lactobacillius* and *Bifidobacterium* species benefit the host by improving gut

efficiency through an increase in nutrient absorption and the acceleration of gut development (Yokota and Coates, 1982; Palmer and Rolls, 1983; Furese *et al*., 1991). The MOS (mainly from the cell wall of yeast) seem to have two types of activity: the adsorption of enteric pathogens and immunomodulation (Newman, 1994). MOS are thought to block the adhesion of pathogenic bacteria to the animal's intestine, preventing the colonisation that may result in disease. They are also considered to stimulate the animal's immune system, thus reducing the risk of diseases (Savage *et al*., 1996; Iji *et al*., 2001; Patterson and Burkholder, 2003).

When the relationship between the oligosaccharide structure and those effects is better understood, it should be possible to design novel prebiotics to maximise the protective effect. Another means of influencing animal health through carbohydrates is via cell-to-cell interactions. Carbohydrates from bacterial cell surfaces function in a variety of ways to influence cell-to-cell communication (quorum sensing) and influence bacterial attachment to the host tissues (Sperandio *et al*., 1999; Newman and Spring, 2004; Fujiya *et al*., 2007). However, while many authors consider prebiotics to be a promising alternative to AGPs for use in animal feeds, the experimental data are still inconsistent and their benefits have not been confirmed by all studies.

17.6.4 Competitive exclusion (CE) and probiotics

The phenomenon by which the normal GI microflora protect the host against invading pathogens is called competitive exclusion (CE). Numi and Rantala (1973) were the first to apply the CE concept to domestic animals, mainly to poultry. That is why CE is also called the 'Numi concept'. These authors observed that the oral administration of gut content, containing viable bacteria and originating from adult pathogen-free birds, could protect young birds against *Salmonella* infections. Numerous articles have been published on this subject in the last 30 years. CE involves preventing the entry or establishment of one bacterial population into the GI tract through the presence of a competing bacterial population that already occupies potential attachment sites. To be able to succeed, a population must be better suited to establish or maintain itself in that environment or must produce inhibitory compounds against its competitors. The adhesion of beneficial bacteria to the GI wall is considered a prerequisite for the CE of pathogens and for the modulation of local and systemic immunological activities. The mechanisms involved in CE by which bacteria operate, i.e. spatial exclusion, micro-environmental alterations, production of antimicrobial substances and epithelial barrier integrity, are very complex and little has so far been discovered about them. Specific adhesin-receptor interactions and non-specific hydrophobic group interactions have been identified as the major mechanisms for adhesion (Ofek and Doyle, 1994). The importance of volatile fatty acids (VFA) as part of the mechanisms of CE has been reported by several researchers in recent years, while others have suggested that

protection is in the first instance a physical phenomenon rather than a process involving synthesis of VFA or other metabolites (Schneitz, 2005). Antibacterial lypophilic factors, antimicrobial compounds (peptides and proteins) and carbohydrates have all been shown to inhibit the adhesion of bacteria to the intestinal cell surface (Coconnier *et al*., 2000).

In animal production, CE uses live, defined cultures of beneficial microorganisms. Gram-positive bacteria of the *Lactobacillus*, *Enterococcus* and *Bacillus* types are used, as are fungi of the *Saccharomyces* (yeast) genus (Revington, 2002). Such cultures are often included in feed as an adjunct to antibiotic therapy in order to reintroduce beneficial flora to the intestinal tract of infected animals. Supporters of CE claim that it is the most effective harmless method available to control GI disturbance in farm animals. This CE concept highlights the role of the GI microflora in ensuring animal health, as well as the importance of favouring the beneficial bacteria in the GI tract, particularly since these have been intentionally introduced into the gut of the animals. One possible means of favouring the proliferation of these beneficial bacteria is the manipulation of substrate availability in the GI tract so that it is able to feed these microflora (*cf*. prebiotics). The modes of administration of live bacterial populations are mainly *in novo,* i.e. individual oral administration, spray (in hatchery for poultry), introduction into drinking water upon arrival on the farm, or spray on first feed (Schneitz, 2005). The treatment is fully biological and leaves no residues. The concept was originally designed for *Salmonella* reduction in growing chickens. Over the years, other pathogens such as pathogenic *Escherichia coli*, *Clostridium perfringens* (Hoang *et al*., 2008) and *Listeria monocytogene* have also been targeted (Mojgani *et al*., 2007) and the practice has been extended to other farm animals too. Preliminary effects on *Campylobacter* and other members of the flora such as viruses and protozoa have been reported (Doyle and Erickson, 2006; Schneitz, 2005). There are currently many commercially available CE products, all of which are mixed cultures derived from the caecal contents and/or mucosa-associated flora obtained from the caeca and/or the gut wall of farm animals.

More recently, an extension of the CE principle has led to more routine administration of probiotics (lactobacilli, bifidobacteria, *Aspergillus*, yeast, etc.) (Fuller, 1991, Rush, 2002). Because of the diversity of the mechanisms of action of probiotics, including immune regulation (Vila *et al*., 2010), they have recently been broadly defined as 'live microorganisms, which when administered in adequate amount, confer a health benefit on the host' (Guarner and Schaafsman, 1998). Probiotics can play a preventive role by competing with pathogens or a curative role by repairing the changes that occur in the GI microflora as a result of stress: they can, at least partially, restore the animal's resistance to some enteric disturbances. This curative effect depends on the level of infection. Moreover, and as a consequence, beneficial effects on body weight gain in farm animals can be achieved by the addition of probiotics in diets (Abaza *et al*., 2008).

However, the factors that affect the colonisation of a given probiotic culture are not clear. Difficulties arise in defining the specific culture that should be used, and in administering the cultures via the feed, since the heat treatments that are often involved in feed preparation (pelleting, for example) are obviously harmful to live cell products. Moreover, it appears that most probiotics do not colonise the intestine but simply pass through. Most of the proposed mechanisms of action (enhancement of the physical and functional mucosal barrier, competitive adhesion to epithelial receptors, reduction of intestinal pH by lactic acid production, modification of bile salt, competitive exclusion) remain hypothetical and much work remains to be done in order to refine the application of this approach (Crevieu-Gabriel and Naciri, 2001). Probiotics also have anti-inflammatory and cytoprotective effects (Canny and McCormick, 2008). In addition, a recent study showed that fermented soybean meal with *Aspergillus* influenced pancreatic and intestinal enzymatic activities and villus height differently according to the age of the animal (Feng *et al*., 2007).

In conclusion, the mechanisms involved in potential beneficial effects of probiotics can broadly be grouped into direct and indirect mechanisms. Indirect mechanisms are the result of the normal microflora altering the physiological response of the host, which in turn affects the interaction between the host and the microorganisms (Rolfe, 1991). Direct mechanisms are exerted by different bacterial populations on each other.

Regarding the use of probiotics in daily practice, a number of diverse factors need to be considered, including the dose, the health status of the farm, production conditions, and husbandry practices. In addition, the inherent anaerobic nature of intestinal bacteria has hindered the commercial development of many effective probiotics. Like AGPs, probiotics appear to have a pronounced effect on farms where the housing and hygiene conditions are suboptimal.

17.6.5 Synbiotics

The bacterial nutrient package will not succeed in the absence of the targeted, beneficial bacteria, and likewise the live microorganism product will not succeed if the environment into which it is introduced is unfavourable. Therefore so-called ‘synbiotic products’, which contain both a probiotic strain and a prebiotic favouring the growth of that probiotic strain, are thought to offer a means of maintaining the correct balance of the GI microflora.

17.6.6 Organic acids

Organic acids, which have antimicrobial activity, are the most frequently used alternative to AGPs. However, the mechanism of their impact on gut microflora is poorly documented. The use of acidifiers in piglet feeds has proven to be beneficial, and organic acids have been used as

Salmonella-control agents in feed and water supplies for livestock and poultry. The success of acidifiers in piglet nutrition is typically a result of the high buffering capacity of the feeds coupled with the limited ability for hydrochloric acid production in piglets. Organic acids are particularly effective as preservatives thanks to their ability to enter a bacterial cell and to acidify its contents, thereby inhibiting microbial enzymes. The energy required to fight acidification is thought to weaken the already distressed bacteria, slowing growth and reproduction. A further benefit is that the acidification of the intestinal tract favours the acid-producing bacteria (lactobacilli, bifidobacteria) and inhibits the acid-intolerant bacteria (*Salmonella, E. coli, Campylobacter* sp.) (Dibner and Buttin, 2002). Dietary acidification brings other benefits, including improvements in gastric proteolysis and protein digestibility, with consequent reduction in growth-restricting microbial metabolites (ammonia), reduction in the digestive pH, and increased pancreatic secretion. The acid anion can also form a complex with minerals, increasing their digestibility. Organic acids also serve as substrates in intermediary metabolism and therefore have an energy content (Revington, 2002). Finally, they have trophic effects on the gastrointestinal mucosa which vary with the formulation of the diet and the type of acid used.

17.6.7 Herbs, spices, essential oils and various plant extracts

Herbs, spices, essential oils and plant extracts have received increasing attention as potential replacements for AGPs. There is evidence to suggest that some of these components have appetite-stimulating, antibacterial, antioxidant, coccidiostatic and even antiviral properties (Langhout, 2000; Wenk, 2003; Abbas and Ahmed, 2010). They have also been claimed to increase digestive enzyme secretion and to lead to improvements in immune functions. These compounds can probably only be effective on a practical scale if they are used in a more concentrated form than that found in nature. These substances are often claimed to be 'all natural'; while this is true, from a feed safety perspective it could be somewhat misleading, as many conventional antibiotics are also 'natural', being produced by *Streptomyces* or *Penicillium* species. It is likely, therefore, that the plant extracts that prove to be most beneficial in the modification of the microbial environment of the intestine will also ultimately be subject to regulatory approval (Revington, 2002).

17.6.8 Specific antibodies

One alternative to antibiotics which offers promising potential involves the inclusion of specific antibodies in feed with the intention of neutralising pathogenic organisms. To provide a straightforward example, hens may be exposed to specific antigens, stimulating their systems to produce immunoglobulins. These immune proteins are then harvested from the eggs and

included in animal feed. The swine industry has used this method with a degree of success; however, there may be some problems with the mode of delivery, since both heat treatments and the digestive processes of the animal obviously have a detrimental effect on the functionality of protein (Revington, 2002).

17.6.9 Bacteriophages

Bacteriophages are viruses that infect bacterial cells and may destroy them by lysis. They can be very specific to certain pathogens. This idea of using bacteriophages in the treatment and prevention of diseases is not new and in fact dates back to the 1920s, but their use was stopped when the first chemotherapeutics (dyes and sulphonamides) and antibiotics became available. Recent work with poultry has suggested that bacteriophages may be useful replacements for antibiotics in the treatment of various diseases, particularly in those cases where pathogens are located on biological surfaces (Biswas *et al*., 2002; Joerger, 2003; Huff *et al*., 2005).

17.7 Conclusions

There are few alternative strategies and agents available today that can apparently offer the same benefits as the AGPs that they purport to replace. Indeed, any replacement for AGPs would have to provide an improvement in feed efficiency that is economically viable. If the AGP substitute does not have antimicrobial properties, other concerns, including the incidence of enteric diseases and airsacculitis, will have to be addressed through the continued use of ionophores, management changes, or both (Dibner and Richards, 2005). Rosen (2004) observed that it was necessary to study the efficiency of numerous candidates simultaneously, because combinations of potential replacements should prove more efficient as alternatives to AGPs. The ban on in-feed AGPs and its consequences for the animal industry is a textbook case for the impact of new regulations. Of course, most European livestock producers totally disagreed with this policy, which was based on the precautionary principle, and stressed the risks for animal health and the transfer of zoonotic pathogens from animals to humans as a consequence of the ban. In addition, reduced animal performance, especially in pig and poultry production, has economic as well as ecological consequences. However, the absence of an 'antimicrobial net' has also led to increased efforts to improve good agricultural practice and hygienic conditions in animal husbandry. The ban on AGPs has also encouraged the scientific community to find alternatives, leading to new concepts and original ideas. However, it is important to emphasise that all these alternatives must be carefully investigated, in order to prevent new feed safety crises and to ensure that these proposed alternatives are indeed safe.

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18

Chemical risk assessment of animal feed

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Abstract: This chapter discusses the risk assessment of chemicals potentially present in feeds, based on the approaches adopted by the European Food Safety Authority, as well as on some significant examples including a coccidiostat, which may also cross-contaminate feeds for non-target species (narasin); a trace element proposed as a nutritional additive, but for which there remain serious uncertainties regarding safety (chromium(III)); a widespread estrogenic mycotoxin (zearalenone); and a persistent organic pollutant with multiple toxicological targets (hexachlorobenzene). Areas in which there are gaps in knowledge and possible areas requiring further investigation are also discussed.

Key words: toxicology, veterinary public health, endocrine disrupters, feed contaminants, feed additives, risk assessment.

18.1 Introduction

The 'farm-to-fork' approach promoted by the European Union (Commission of the European Communities, 2000) requires the assessment and control of major components of the food production chain, with emphasis on primary production. Feeds must satisfy the nutritional requirements of the relevant animal species in the industrialized world. Feed composition is also expected to support the cost-effective production of meat, eggs and milk from selected, specialized breeds (Thong *et al*., 2004). Most importantly, feed quality is essential to ensure both the welfare and safety of farm animals and the safety of their products (Mantovani *et al*., 2006). Food-producing animals depend on the quality of their living environment, and feed contamination by environmental pollutants has resulted in rapid alerts more than once in industrialized countries, e.g., the several instances of feed and/or pasture pollution by polychlorinated biphenyls (PCBs) (La Rocca and Mantovani, 2006).

Such episodes have prompted greater attention to the environment–feed–food chains, from both regulatory and research standpoints. Besides

ingredients (and their possible contaminants and/or undesirable components), feeds utilized in intensive farming require the use of a diverse range of additives, much like human foods throughout the industrialized world. In fact, feed additives account for the bulk of chemicals used in animal production, including a large and heterogeneous group of compounds used for nutritional (vitamins, trace elements), zootechnical (coccidiostats, probiotics, etc.), sensory (e.g., colouring agents) or technological (e.g., preservatives) purposes (Mantovani *et al*., 2006). This chapter provides an overview of the risk assessment of chemicals present in feeds, as both additives and contaminants, in accordance with the approaches laid down by the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/). This overview is supported by several examples including a coccidiostat (narasin); a trace element proposed as a nutritional additive (chromium(III)) for feed; a mycotoxin (zearalenone) and a persistent organic pollutant (hexachlorobenzene) as contaminants. Areas in which there are gaps in knowledge and possible areas requiring further investigation will be also discussed.

18.2 Risk assessment of feed additives and contaminants

European feed additive risk assessment is based on three main principles (Commission of the European Communities, 2001): (a) pre-market authorizsation; (b) positive list principle; and (c) an assessment of the possible effects on human and animal health as well as on the environment. Since the establishment of the European Food Safety Authority (EFSA) in 2003, the risk assessment of feed additives is the task of the panel on additives and products or substances used in animal feed (FEEDAP) (http://www.efsa.europa.eu/en/panels/feedap.htm).

Assessing a feed additive is a complex process that requires a comprehensive, multidisciplinary approach involving toxicology, veterinary medicine and animal nutrition. Compounds intended for deliberate addition to animal feed must be safe for animals and consumers at the intended dose levels. Clear parameters are required in order to regulate the safe use of the additive, such as:

- The maximum concentration that can be used in feeds (which might be lower than the intended one).
- The margin of safety between the maximum concentration and the level at which signs of intolerance appear in farm animals. Information should also be provided to assess whether the claim of efficacy at the intended use level(s) is credible.
- In the case of xenobiotics such as coccidiostats, a toxicologically derived definition of an acceptable daily intake (ADI) as well as of the maximum residue limits (MRLs), which ensure that the exposure of consumers does not exceed the ADI. The approach used is similar to that used for veterinary drugs (Mantovani and Macrì, 2002).

- Where nutrients (trace elements, vitamins) have been added, tolerable upper intake levels (UL) are used to assess consumer safety. A UL is the highest level of daily nutrient intake at which there is likely to be no risk of adverse health effects for almost all individuals in the general population (Vanderveen, 2006). Tissue deposition occurring from the use of the feed additive, together with background dietary levels and other intake sources (e.g., supplements) should also be considered when assessing the consumer exposure risk. No MRL can be defined, since it would be extremely difficult to distinguish, under practical conditions, the contribution from feed additives and the natural background (see, e.g., EFSA opinions on iodine salts or vitamin A: European Food Safety Authority, 2005a, 2008a respectively).
- Last but not least, parameters relating to the tolerance in animals and deposition in edible products have to be established for each major species for which the additive is intended.

The safety assessment of a feed additive also incorporates the potential impact on the safety of workers who are exposed to the additive (e.g., people involved in mixing the additive with the feed) and includes estimation of inhalatory exposure, an assessment of local effects (skin and eye irritation, dermal sensitization) and possible proposals for risk phase and labelling. Enzymes and micro-organisms are not usually a cause for concern regarding residues, but, in the absence of proper data, are considered by default to be respiratory sensitizers, due to the potential exposure of workers to protein products (see, for example, European Food Safety Authority, 2008b).

Ecotoxicity is another major issue in risk assessment; in fact, mass use of feed additives in intensively farmed animals may lead to a significant environmental exposure through animal excreta (Wollenberger *et al*., 2000). The problem is relevant not noly in xenobiotics: e.g., the case of copper (Cu), which is an important dietary requirement in pigs but is highly toxic to small ruminants grazing on pastures contaminated by pig excreta (Kerr and McGavin, 1991). The FEEDAP panel has put forward a tiered approach to the ecotoxicological assessment of feed additives for aquatic and terrestrial environments. The requirement for acute or long-term ecotoxicity tests is triggered by exposure modelling (European Food Safety Authority, 2007b).

The assessment of substances that may occur as undesirable components or environmental contaminants in feeds is carried out by the panel on contaminants in the food chain (CONTAM, http://www.efsa.europa.eu/en/panels/contam.htm). In many cases, e.g., most mycotoxins and plant-derived compounds (European Food Safety Authority, 2004b, 2008a), feed contaminants represent a risk mostly for farm animal welfare. In other instances, however, they may be a major topic for veterinary public health. For instance, feeds can be a major vehicle for the presence of PCBs and other persistent lipophyllic pollutants in the human diet (La Rocca and

Mantovani, 2006). Another cause for serious concern is the role of aquaculture feeds in the contamination of the human diet with PCBs, dioxins and methylmercury, since high levels of contaminants may reduce the recognized beneficial actions of nutrients such as omega-3 which are abundant in fish (European Food Safety Authority, 2005b). Indeed, the interactions between contaminants and nutrients are an extremely important topic for food safety risk-to-benefit assessment, yet relatively little is yet known about them (Baldi and Mantovani, 2008). Interestingly, a few chemicals have been assessed both as additives and as contaminants. Coccidiostats, in particular, have been assessed by FEEDAP according to their intended use and by CONTAM as regards the possible cross-contamination of feeds for non-target species (European Food Safety Authority, 2004a, 2007a). The stepwise assessment of feed additives cannot be applied to contaminants, so a case-by-case approach is adopted. Critical issues include the characterization of toxicological hazards and the possible pathways of feed contamination as well as the transfer of the parent compound or metabolites to foods of animal origin. Thus, besides the possible recommendations of maximum tolerable levels in feeds, a major target for risk assessment would be to pinpoint potential situations of higher exposure that may require measures for risk management. To this end, the work undertaken by EFSA on undesirable substances has been considerable: from 2004 to April 2009 the CONTAM panel has completed 30 risk assessments, including natural plant products (e.g., gossypol), persistent organic pollutants (e.g., DDT), trace elements (e.g., arsenic), and mycotoxins (e.g., aflatoxin B1). In most cases, no risks to animal health and no concern for health risks related to carry-over to edible products were identified; however, potential adverse effects on animal health at current exposure levels could not be excluded in some instances, especially for natural plant products (e.g., gossypol in sheep). Moreover, the CONTAM panel issued recommendations to reduce the presence of several persistent organic pollutants (e.g., camphechlor) in edible tissues and products. The need for further research on carry-over from feed to foods of animal origin in order to refine exposure assessment was identified (European Food Safety Authority, 2009a).

18.2.1 A coccidiostat: narasin

During a general evaluation of coccidiostats used as feed additives in Europe for compliance with regulatory requirements, FEEDAP delivered their opinion on the efficacy and safety of Monteban®, a product containing not less than 10% of narasin as the active substance. Monteban was intended for chickens for fattening at levels of 60–70 mg/kg complete feed (European Food Safety Authority, 2004a). Narasin is a polyether carboxylic ionophore; like similar compounds, narasin changes ion gradients and electrical potentials in cell membranes, thus impairing cellular function and the

metabolism of coccidia. The adverse effects in animals and humans stem directly from the compund's pharmacodynamic properties.

Literature data published in the 1990s indicate that narasin is effective as a coccidiostat for broiler chickens at the maximum dose level authorized in the EU of 70 mg/kg complete feed. More recent field studies, however, are not available to prove that the compound is still effective at that intended feed concentration. Indeed, the development of resistance against coccidiostats, including narasin, is now well recognized. The FEEDAP did not, however, find any evidence that a special problem of an unusual resistance to narasin should be expected. The levels used in feeds for the treatment of coccidiosis are also effective in the prevention of necrotic enteritis in chickens. The compound has also shown antibacterial activity against Gram-positive bacteria, while Enterobacteriaceae are resistant; even so, no cross-resistance to other antimicrobials is yet recognized, except to salinomycin.

The low tolerance of farm animals to narasin may be a problem. Toxicity occurred in turkeys and rabbits at feed concentrations lower than the maximum level for chickens for fattening. Dogs, horses and cattle may also be particularly sensitive. Tolerance tests in chickens for fattening showed a small margin of safety (about 1.4). Signs and lesions in target and non-target animals are consistent with the mode of action of polyether ionophores, including dyspnoea, lung oedema, liver cell necrosis and muscle fibre damage. Moreover, the simultaneous use of some medicinal substances (i.e. tiamulin) should be avoided, as their adverse interactions with narasin are well known.

Pharmacokinetic data on the effects of narasin on chickens is incomplete, but rapid excretion is indicated. The main metabolic pathway is similar in chickens and rats and involves oxidative processes. Narasin metabolites in tissues and excreta are qualitatively similar, implying that toxicological tests in rats appropriately assess the same metabolites present in edible tissues. The liver is the target tissue for total residues; however, unchanged narasin disappears quickly from tissues, while it is somewhat more persistent in the chicken's skin/fat, where it represents the major residue fraction. Each of the many narasin metabolites represents less than 10% of the total tissue residues. Using a pragmatic approach, therefore, the FEEDAP retained the parent compound as marker residue and skin/fat as marker tissue. Narasin may concentrate in the egg yolk (Rokka *et al*., 2005) but the compound is not intended for use in laying hens. As with other coccidiostats, many toxicological studies on narasin were old and not fully compliant with current quality standards. The critical toxicological effects were focal degeneration of skeletal muscles, including the diaphragm, and peripheral neuropathy in dogs. The no-observed-adverse-effect-level (NOAEL) of 0.5 mg kg^{-1} bw per day seen in the one-year dog study was used to set the ADI of 5 μg kg^{-1} bw (equal to 300 μg per day for a person of 60 kg body weight). A MRL of 0.05 mg narasin kg^{-1} for all tissues was defined with a withdrawal

time of one day; this is sufficient to keep consumer exposure below 20% of the ADI, even using highly conservative, default figures of human consumption (e.g., 300 g/day of chicken meat). As regards occupational safety, the whole product (Monteban) can cause irritation to the eyes, but not to the skin. Inhalation studies in dogs showed that narasin is potentially highly toxic by the inhalation route, even more so than by the oral route.

There is also a sensitivity potential through skin contact and by inhalation, but the product is formulated as granules with a low dusting potential. For this reason, it is not expected that workers will be exposed by inhalation to toxic levels of narasin dust as a result of its handling. Nevertheless, FEEDAP recommended the use of appropriate personnel protective equipment for the workers.

Data were insufficient for an adequate environmental risk assessment, but based on the available information on the toxicity, fate and behaviour of narasin, FEEDAP could not rule out the possibility that use at the recommended dose range poses a risk for soil organisms. There was insufficient data to assess the risk to the aquatic environment and secondary poisoning of upper food-web levels (birds and mammals). Therefore, although an ADI and MRL could be defined, FEEDAP noted a deficiency of data on both efficacy and the environmental impact of narasin, as well as potential concern for tolerance levels and occupational safety that need appropriate management.

As previously mentioned, coccidiostats were also assessed by the CONTAM panel for their potential cross-contamination of non-target feed (European Food Safety Authority, 2007a). Indeed, such an assessment is fully justified by the concerns for toxicity in non-target species of compounds like narasin, as well as by the likelihood of exposure under practical conditions. During the production of mixed feeds, a certain percentage of a coccidiostat-containing feed batch remains in the production circuit and these residual amounts can contaminate subsequent feed batches intended for non-target animal species. For narasin in particular, toxicological data on non-target domestic animals indicates the high sensitivity of some species. Nevertheless, adverse effects are not expected to occur in non-target animals if cross-contamination is kept ≤10% of the maximum amount permitted in the feed of target animals (i.e., ≤7 mg/kg complete feed). The assessment is based on the long-term toxicological effects in dogs, the most sensitive laboratory animal species, where a NOAEL of 0.5 mg/kg bw was identified, based on the induction of neurotoxicity in a one-year feeding trial. Assuming a feed intake of approximately 50 g/kg bw per day, which is applicable to most monogastric food-producing animals, a 10% cross-contamination level would result in a daily exposure of 0.35 mg/kg bw per day, which is still below the one-year NOAEL in the most sensitive species (European Food Safety Authority, 2007a). Since narasin does not bioaccumulate, cross-contamination does not raise serious concerns for consumer exposure. According to kinetic studies, hens' eggs and pigs' liver show the

highest levels of narasin residues in edible tissues products of non-target animals. Even using highly conservative, default figures for human consumption (e.g., 50 g/day of eggs), if cross-contamination is kept up to a level of 10%, the resulting human exposure would be below 50% of the ADI. The likely low frequency of significant cross-contamination by narasin adds further reassurance to the estimation that no risk for consumers is expected with a cross-contamination level up to 10%.

18.2.2 A trace element and its organic form: chromium(III) and Cr(III)-methionine

The assessment of Cr-methionine as a nutritional additive for all species was completed in 2009 by FEEDAP; Cr was not allowed as a trace element in farm animal nutrition, thus, the specific assessment of the organic chelate of the trace element required a preliminary, comprehensive evaluation of the possible role of Cr(III) as a nutritional additive as well as of the related risks (European Food Safety Authority, 2009b). Cr(III) naturally present in feed materials derives mainly from mineral sources and only to a limited extent from plant sources. Cr(III) has a recognized endocrine-metabolic action, enhancing the insulin-dependent glucose entry into the cells as well as, albeit with a lower level of evidence, modulating immune response, leptin balance and lipid metabolism (Mantovani *et al.*, 2009). However, no symptoms of Cr(III) deficiency have been demonstrated in either animals or humans, meaning that no clear evidence for a possible role as essential nutrient and no requirements for Cr(III) in farm animals can be established. Cr(III) cannot, therefore, be considered as a nutritional additive, despite a wealth of studies intended to demonstrate its favourable effects in farm animals. These favourable effects are especially evident in animals under stress conditions and depend also on a natural Cr(III) background in the feed, the sources and levels of supplementary Cr and the presence of other dietary factors, including other trace elements such as Fe, Mn, V and Zn (European Food Safety Authority, 2009b). Similar limitations and uncertainties prevent an assessment of the maximum tolerable levels of supplemental Cr(III) in feed based on the tolerance in farm animals. It is also difficult to assess the bioavailability of Cr(III) due to the lack of established biomarkers. The toxicology of Cr(III) is also not yet fully clarified. Cr(III) is much less toxic than Cr(VI), an environmental contaminant and an established carcinogen, but recent literature suggests a possible genotoxic potential *in vivo* (Kirpnick-Sobol *et al.*, 2006; Medeiros *et al.*, 2003). Undesirable endocrine-metabolic effects may also be considered (Mantovani *et al.*, 2009). It is noteworthy that the Scientific Committee on Food was unable to establish an upper tolerable limit for humans for Cr(III), due to inadequate information (European Commission, Scientific Committee on Food, 2003). Taking into account concerns and uncertainties, the FEEDAP panel considered it prudent to avoid any additional exposure of consumers

resulting from the use of supplementary Cr in animal nutrition. Consumer carry-over from Cr(III) in feeds is also difficult to assess since the available data do not show a consistent pattern in tissue deposition and due to significant analytical uncertainties (European Food Safety Authority, 2009b). The consumer background dietary intake of Cr(III) is likely to be around 0.1 mg/day and is not expected to exceed 0.3 mg/day. The contribution of foodstuffs of animal origin from unsupplemented Cr(III) animals to the background dietary intake for adults is estimated to be in the range of 16–26%; the main contributors include offal, followed by muscle (including fish) and eggs (European Commission, Scientific Committee on Food, 2003; European Food Safety Authority, 2009b; Leblanc *et al*., 2005). However, no reliable data are available to assess the additional consumer exposure resulting from the use of supplementary Cr in feeds. Finally the FEEDAP panel noted that concerns about Cr(III) toxicity, and especially genotoxicity, should also be considered when assessing user safety. Occupational exposure in the feed industry should therefore be kept to a minimum.

Against this rather unfavourable background, FEEDAP carried out a specific assessment of the organic trace element compound, Cr(III)-methionine; the compound actually contains 3% Cr(III) and is a chloride salt of a hexacoordinate chelate complex of a Cr(III) atom with three methionine molecules. The intended supplementation level was 0.4–1.6 mg/kg complete feed as Cr(III). Overall, dietary Cr is poorly absorbed by animals (<1% of the ingested dose): limited evidence suggests that organic forms of Cr(III) are better absorbed, such as Cr-nicotinate, Cr-picolinate or Cr-yeasts, in the range 1.5–10% of the ingested dose. Cr(III)-methionine was considered a bioavailable source of Cr(III), based on several biomarkers related to glucose metabolism, as the main biological target of Cr(III), namely increased glucose clearance rate in pigs and beef cattle, reduced plasma glucose in horses and dairy cows, and reduced insulin in horses. A tolerance study in pigs did not show any significant effect, whereas one feeding study on cows suggested a negative effect on milk production at near-use levels. The limited data did not allow for a conclusion that the enhanced bioavailability of Cr(III)-methionine was of no consequence for farm animal safety. Genotoxicity was a point requiring clarification: due to the relationship between the effect and bioavailability, Cr(III) is the likely ultimate intracellular form of carcinogenic Cr(VI); Cr(III) itself has very low intracellular accessibility, but this might be significantly enhanced in organic forms (Nguyen *et al*., 2008). Indeed, a recent study showed a markedly higher reactivity with cell proteins of the organic form of Cr(III) picolinate compared to the inorganic form. The question has not been answered by the studies provided on Cr(III)-methionine; although they did yield negative results, they were considered inconclusive since the endpoints typical of Cr(VI) genotoxicity (e.g., oxidative DNA damage, DNA adducts) were not specifically assessed. Tests showing Cr(VI) genotoxic effects in direct comparison with Cr(III)-methionine should have been provided, instead. Finally,

the critical issue of consumer exposure assessment could not be resolved due to the inadequacies of the studies performed. It was only possible to assess that no significant carry-over occurred in bovine milk; however, milk itself is not a significant dietary source of Cr(III) compared to offal, meat or eggs (European Commission, Scientific Committee on Food, 2003; European Food Safety Authority, 2009b). Overall, the data were inadequate for dealing with the main questions associated with the enhanced bioavailability of Cr(III), such as genotoxicity and tissue deposition; thus no conclusion could be made as to whether the use of Cr(III)-methionine in farm animal feeds would result in any different concern for consumer safety compared to other Cr(III) sources. As previously discussed, Cr(III) and Cr(III)-methionine cannot be considered as nutritional feed additives due to the lack of their demonstrated essentiality; however, the need for recent and relevant data on consumer exposure and safety remains, especially considering the other potential uses of Cr(III), e.g., as production enhancers, in farm animal nutrition.

18.2.3 A mycotoxin: zearalenone

Zearalenone is a mycotoxin produced by several field fungi, including *Fusarium graminearum* and *Fusarium culmorum* (European Food Safety Authority, 2004b). The toxin is common in maize and maize products, but can be found in soybeans and various cereals and grains, as well as in their by-products. Moreover, zearalenone seems to occur on grass, hay and straw, resulting in additional exposure of animals from their roughage and bedding. Consequently there is significant likelihood of exposure to zearalenone for most farm animal species. *Fusarium* spp. also produce other toxins, particularly the immunotoxic deoxynivalenol (European Food Safety Authority, 2004c) and the neurotoxic fumonisins (European Food Safety Authority, 2005c). The co-occurrence of zearalenone with other mycotoxins is frequent, and exposed animals may show mixed clinical signs; however, since the mode of action of zearalenone is different from other *Fusarium*-derived toxins, additive effects between toxins are unlikely. In mammals zearalenone is a powerful endocrine disrupter; it interacts with oestrogen receptors, inducing an apparent hyperoestrogenism with related effects, such as reduced fertility (Minervini and Dell'Aquila, 2008). Besides the primary, endocrine effect, zearalenone may induce liver and immune toxicity, as well as DNA damage, in both laboratory and farm animals; such effects could also be related to the oestrogenic action (Zinedine *et al.*, 2007). Among farm animals, young female pigs are the most sensitive species; bile concentration of the parent compound and its metabolites, α- and β-zearalenol, is the best biomarker of exposure in pigs (Goyarts *et al.*, 2007). Rather limited data indicate that sheep are the next most sensitive farm animal after pigs, followed by cattle. Poultry (chicken and turkey), meanwhile, are quite resistant to the hormonal effects of zearalenone. Insufficient data exist on some

so-called 'minor' species (nonetheless important in some countries) such as the rabbit. Due to the rapid biotransformation and excretion of zearalenone in animals, secondary human exposure due to carry-over from feeds to foods of animal origin appears to be low (European Food Safety Authority, 2004b; Goyarts *et al*., 2007). It is noteworthy that low feed-to-food carry-over is a feature shared by other undesirable endocrine-active compounds of plant origin; a well-known example is goitrogenic glucosinolates derived from *Brassica* spp., which may impair animal welfare and production but are considered of limited significance for the safety of foods of animal origin (European Food Safety Authority, 2008c). The available evidence indicates that animal products generally contribute only marginally to human dietary exposure to zearalenone; nevertheless, zearalenone is a real risk for farm animal welfare, as outbreaks have been recorded, especially in pigs (European Food Safety Authority, 2004b; Zinedine *et al*., 2007). Good feed manufacturing and farming practices are the best way to prevent the hazard. Control of the storage conditions of feed ingredients at risk (e.g. corn) may substantially reduce the growth chances of zearalanone-producing *Fusarium* spp. The monitoring of feed will support prevention measures, allowing exposure assessment in due course, to check, for example, for any effect on contamination due to climate changes (Miraglia *et al*., 2009). Further research is also needed to establish safe levels of exposure for zearalenone in feed materials for farm animal species such as rabbits and small ruminants.

18.2.4 A persistent organic pollutant: hexachlorobenzene

Hexachlorobenzene (HCB) was introduced as an agricultural pesticide in 1945, and was banned in 1981 for agricultural use in Europe (European Food Safety Authority, 2006). The current exposure to HCB is, therefore, mainly due to its persistence and bioaccumulation. Indeed, HCB is quite volatile, highly lipophylic and among the more persistent environmental pollutants. As a result, it can be transported over long distances and is bioaccumulated in fatty tissues. For these reasons HCB is included in the international protocols on persistent organic pollutants (United Nations Environment Programme: http://www.chem.unep.ch/pops/). Nevertheless, current release sources do exist, as a by-product of incomplete incineration processes, leakage from old dump sites, and inappropriate manufacturing, including waste disposal, of a number of chlorinated compounds, such as solvents and pesticide formulations. Overall, HCB is ubiquitously present in environmental and biological samples worldwide.

HCB is readily absorbed by both humans and animals. Whilst acute toxicity is low, medium and long-term exposures have been associated with a number of negative health effects. The liver is one major target organ, with porphyria and the induction of cytochrome P-450 enzymes appearing as the primary biochemical effects of HCB. Other important effects are immune dysregulation, involving macrophage and pro-inflammatory

activation (Ezendam *et al*., 2005), as well as ovarian toxicity and apparent antioestrogenicity with reduced circulating oestradiol and tissue oestrogen receptors (Alvarez *et al*., 2000). While HCB is negative in most genotoxicity tests, the metabolism of HCB can result in the formation of reactive intermediates such as epoxides, which can covalently bind with proteins and DNA. HCB induces liver tumours in female rats, while male rats appear much less vulnerable: the mode of action may be related to long-term alterations in intercellular gap junctional communication. Noticeably, gender-related susceptibility to liver tumours is not dependent on ovarian function, as it is observed also in ovariectomized rats (Plante *et al*., 2002).

In farm animals, HCB has a low acute toxicity, thus available data focuses on the medium- to long-term effects. Feeding exposure to HCB elicited liver effects in pigs and lambs and offspring mortality in mink, with NOAELs in the 1.0–0.1 mg/kg feed range, equivalent to a ≤0.05 mg/kg bw range. Chickens and rainbow trout apparently experienced adverse effects only at feed concentrations higher than the 5–10 mg/kg range (European Food Safety Authority, 2006). In major farm animals the main issue is the feed-to-food carry-over. The following were reported in the CONTAM opinion: transfer of HCB to milk in the range of 2.0–10.5% of the ingested dose; transfer into eggs in the range of 1.3–5.5% of the ingested dose; in fish about 80–90% of the ingested dose can be retained in the organism; for pigs and chickens, the accumulation ratio was estimated to range from 8–11% and 11–30%, respectively (European Food Safety Authority, 2006). The main exposure factors for farm animals were related to the use of animal-derived oils in feeds (as in aquaculture) and also to vegetable fats and pastures from highly polluted areas. Like farm animals, human dietary exposure to HCB is linked to certain food commodities. Fatty fish and fish-derived products, particularly fish oils, generally contain the highest levels of HCB. High levels may occasionally be found in lipid-containing plant products, such as pumpkin seeds and vegetable oils, from contaminated areas. One special instance of dietary exposure to HCB, and other persistent organic pollutants (POPs), is breastfeeding. The maternal body burden related to previous dietary exposure is a major factor in the POP contamination of human breast milk (Campoy *et al*., 2001); whilst the beneficial effects of breastfeeding are not in question, this issue serves to reinforce the relevance of securing safe feeds, and therefore safe foods of animal origin. It is also important to work towards the new concept of 'sustainable food safety', targeting the prevention of risks for the next generation(s) (Frazzoli *et al*., 2009). Overall, feed transfer to foods of animal origin appears to play an important role in human dietary exposure to HCB. The HCB body burden has been shown by some human biomonitoring studies: for instance, among several POPs only HCB and the DDT metabolite DDE were consistently present in an Italian study on adult subjects (Attard Barbini *et al*., 2004). Nevertheless, the available data show a considerable decline of HCB presence in foods during recent decades; for instance, a constant decrease of the concentration of HCB and

other POPs was detected in the adipose tissue of cattle and especially pigs over the period 1991–2004 (Glynn *et al*., 2009). The suggested health-based guidance value for HCB, of 170 ng/kg bw per day, dates from 1997, thus it might not account completely for the effects of potential concern (endocrine disruption and immunoxicity) that have been pointed out since then. Such a reference value does, however, show safety margins in the 50-fold to 100-fold range compared with more recent dietary exposure estimates (excluding breastfed infants) (European Food Safety Authority, 2006). Targeted monitoring programmes of the environment and feed ingredients are likely to reduce residual concerns relating to HCB.

18.3 Future trends

It is necessary, during a toxicological risk assessment of feed additives, to take into account some more general trends in food safety. The first issue is the demand for food production chains to provide better-quality foods which also have a lower environmental impact. Feed components, ingredients and additives, should all support animal welfare according to the modern conditions of farm animal rearing. These conditions, and the related welfare assessment, might be considered complex when including, for example, highly genetically selected breeds, 'organic' farming, and promoting and qualifying so-called 'minor species', such as dairy sheep farming and non-salmonid aquaculture. Supporting animal welfare will help to reduce the burden of farm animal diseases and the consequent use of veterinary drugs and will ultimately result in a safer food chain. In terms of risk assessment, therefore, tolerance in target species should be measured through appropriate welfare markers rather than be limited to zootechnical performance.

Creating a realistic exposure assessment which considers the risk for potentially vulnerable population groups is a major issue for consumer safety. Whenever appropriate, the FEEDAP panel has already accounted for age- and gender-related differences in consumption patterns and vulnerability to specific substances, for example in its opinions on the use of selenized yeast and vitamin A in feed, where the assessments considered, respectively, the dietary exposure of small children and the greater vulnerability of post-menopausal women (European Food Safety Authority, 2008a, 2011). It is worth noting that, in both cases, the assessment ended in a recommendation to reduce the total maximum permitted levels of the additive in feeds.

A further aspect requiring careful evaluation is whether feed additives might actually promote consumer health. Indeed, it might be recommended that the risk assessment of feed additives should also include the characterization of possible changes in composition of edible tissues/products. A substantial modification of fatty acid composition or trace nutrient content might be as relevant to consumers as the presence of residues that can be

managed by setting appropriate withdrawal times. It is possible that feed additives could be used specifically to improve the nutritional quality of foods. Such a possibility should not be excluded, provided that some caveats are adhered to, e.g. that the nutritional benefit to humans should be substantiated and that the use of the feed additive should exceed the nutrient's UL even for high consumers of certain food commodities.

Feed contaminants are, in principle, an unavoidable problem, but efforts should be made to reduce the contamination risks in vulnerable areas. Many potential compounds are either natural substances or outdated chemicals, as shown in the two examples zearalanone and HCB discussed above in detail (European Food Safety Authority, 2004b, 2006). This means that risk assessments cannot avail of up-to-date dossiers, nor are there any applicants to refer to in order to obtain further data. Instead, risk assessment must rely on scientific literature (which may be scanty for 'unfashionable' topics) and on results from monitoring plans (which may be unavailable for contaminants not included in monitoring). In the case of most feed contaminants, therefore, there is a general need for additional carry-over data and targeted toxicological studies aimed at covering data gaps, such as effects and dose–response data in minor species or updating guidance values (see, for example, European Food Safety Authority, 2006). More specifically, for vulnerable production chains it is important to develop feed sources that would be less liable to contamination.

A telling example is farmed fish (European Food Safety Authority, 2005b). The contamination levels (methylmercury, PCBs, dioxins) of farmed fish do not differ significantly from those of caught fish, a fact attributable primarily to the use of fish oil and fish meal in aquaculture feeds. Nevertheless, fish makes an important nutritional contribution to the diet; in particular, the intake of omega-3 fatty acids from fish is generally recognized as beneficial to cardiovascular health and brain development. Therefore, improving the production of safe fish is also a public health goal for which the improvement of feeds is a key factor. Accordingly, such research initiatives as the European integrated project AQUAMAX (http://www.aquamaxip.eu) are seeking to develop novel, vegetable-based feed ingredients which are less vulnerable to bioaccumulating contaminants.

In conclusion, the risk assessment of compounds present in feeds shows many specific features related to multi-faceted interfaces between toxicology, animal and human nutrition, pharmacology and chemistry. There are, however, currently several data gaps, which indicate stimulating issues for future further research.

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19

Safety of genetically modified (GM) crop ingredients in animal feed

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Abstract: The cultivation of GM crops has steadily increased since their introduction more than a decade ago. GM crops are considered a separate category of foods and feeds, for which market approval is needed in many countries, including a pre-market safety assessment. This chapter discusses the regulatory background in various countries, and the issues surrounding the safety of GM crops to be used in feed or food. Safety assessments follow an internationally harmonized approach as laid down, for example, in Codex Alimentarius guidelines for foods, which can be translated to the feed situation as well. This approach is based on a comparative assessment of a GM versus a non-GM crop, the latter having a *history of safe use*. Differences thus identified will be at the focus of the subsequent safety assessment. While international consensus exists on this basic approach, there still are differences between countries regarding their legal requirements for labelling, traceability, and detection of GM products. Regulatory authorities are currently reviewing their approach towards the low-level presence in imported feed of unauthorized GM crop varieties that have been authorized elsewhere. As feed, unlike food, is not covered by Codex, harmonization is also desirable in this respect.

Key words: animal feed, crop biotechnology, genetic modification, feed safety, international harmonization.

19.1 Introduction

In the mid-1990s, genetically modified (GM) crops were introduced into the market on a large scale for the first time. Since then, the cultivation of GM crops has steadily increased in terms of acreage planted with these crops, amounting to 148 million hectares worldwide in 2010 (James, 2011). This acreage is not evenly distributed around the globe, with, for example, the United States of America (USA), Argentina, and Brazil accounting for a majority of the production worldwide. Most of the current GM crops have

particular agronomic traits, in particular herbicide resistance and pest resistance, and combinations thereof when multiple genes have been combined within a variety (James, 2011). Herbicide resistance may enable different weed management practices, allowing herbicides (i.e. weed-killing substances) to be sprayed over the crop itself, replacing the spraying of herbicides between crop rows or mechanical weed removal. Without herbicide resistance this might not be possible as herbicides can be toxic to crops as well as weeds. Pest resistance is usually acquired through the introduction of small quantities of insecticidal proteins, such as those designated 'Cry proteins', which are naturally present in crystal-like inclusions in the soil bacterium *Bacillus thuringiensis*. Preparations of *B. thuringiensis*, also referred to as 'Bt', have been used for decades as a natural biopesticide in agriculture and forestry. The Cry proteins introduced into insect-resistant GM crops specifically exert toxicity on a particular insect species but not on animals and humans.

In the legislation of many countries, GM organisms (GMOs) including GM crops are considered a separate category of foods or feeds, for which marketing approval will be needed in advance of commercialization. The procedure towards approval contains a scientific stage in which the safety of the GMO for human and animal health is assessed by risk assessors (i.e. scientists). The outcomes of the scientific stage inform risk managers (for example, national policy makers) whether or not the approval of the pertinent GMO would raise any (potential) safety concerns. This chapter will discuss the regulatory background for GM crop approval and the issues surrounding the safety of GM crops to be used in feed or food that are commonly considered during the safety assessment procedure.

19.2 Regulatory context for genetically modified (GM) crops to be used in feed

As noted previously, the marketing of GM crops requires regulatory approval in many countries. This section will provide examples of the regulatory context for GM crops in various important regions for trade and/or cultivation of GM crops and derived products that can be used for food, namely the European Union (EU), the USA and Canada. While it is realized that there are more countries with regulatory requirements that pertain to GMOs, the examples selected are considered to provide a good insight into the different regulatory approaches that are followed by national governments.

Table 19.1 provides an overview of the GM crops that have been authorized (or for which consultations have been completed in the USA – see the pertinent section below) in the EU, USA and Canada. In total, 17 crop species, including major commodity crops such as canola, cotton, maize, and soybean, are included in this list of authorizations or completed

Table 19.1 Authorized uses (EU, Canada) and completed consultations (USA) for GM crops to be used as food or feed[a]

Crop	Number of authorizations/completed consultations					
	USA		Canada		EU	
	Food	Feed	Food	Feed	Food	Feed
Alfalfa	2	2	2	2		
Canola (oilseed rape)	16	16	11	10	4	4
Cantaloupe	2	2				
Cotton	20	20	15	12	4	4
Creeping bentgrass		1				
Flax	1	1	1	1		
Maize	33	34	23	22	11	11
Papaya	2		1			
Plum	1					
Potato	28	28	20	18	1[c]	1
Radicchio	3					
Rice	2	2	1	1		
Soybean	9	9	7	7	3	3
Squash	2		2			
Sugar beet	3	3	2	2	1	1
Tomato	7[b]		4[b]			
Wheat	1	1				
Grand total	132	119	89	75	24	24

[a] Status according to the following sources of information accessed on 4 March 2011: FDA (2011), CFIA (2011), Health Canada (2011), European Commission (2011). No 'stacks' (crossings) of previously authorized GM crops have been included in the counts featured in the table. This is because no specific requirement for pre-market authorization of stacks of previously approved GM crops exists in the USA or Canada, contrary to the EU, where the pre-market authorization of stacks is mandatory.

[b] One of these tomatoes is the Flavr Savr tomato which encompasses a range of events, of which the specific event has not been specified in the decision document with the authorization/completed consultation.

[c] In the EU, GM starch potato EH92-527-1 is allowed in food at a maximum level of 0.9%.

consultations. Whereas the total number of authorizations in the EU may appear to be relatively low, the chronology of these authorizations for food and feed as depicted in Fig. 19.1 shows that the higher numbers for the USA and Canada are mainly associated with peaks in the 1990s. The annual number of authorizations appears to have been more similar in the three legislations during the last few years (Fig. 19.1).

19.2.1 Regulatory context in the EU

The European Union currently comprises 27 member states, plus a number of 'outermost regions', each of which has to implement EU regulations in its own national legislation. This also holds true for EU legislation on

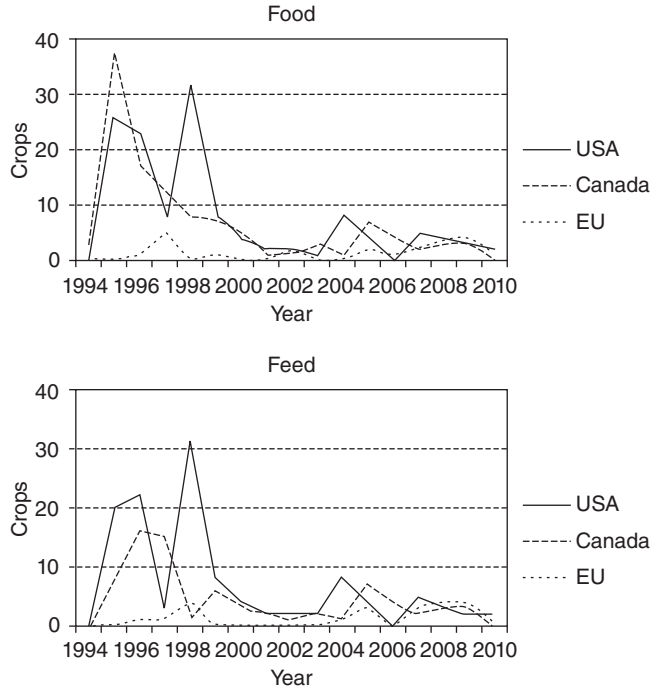

Fig. 19.1 Chronology of authorizations (Canada, EU) and completed consultations (USA) for GM crop events to be used as food (above) and animal feed (below). Data are from the same sources as for Table 19.1 (FDA, 2011; CFIA, 2011; Health Canada, 2011; European Commission, 2011).

GMOs. There are various EU regulations that pertain to GMOs, including regulations on their food or feed applications, environmental release (including, for example, import and cultivation), and labelling.

For the marketing of a GMO and its derived products as a food and/or feed, Regulation (EC) 1829/2003 specifies that the applicant for an approval shall make an application (EU, 2003a). This application shall be accompanied both by a dossier containing safety data and by a method (including reference materials) for the specific detection of the pertinent GMO. The application is to be handed in to one of the competent authorities within a given member state of the EU, which will then forward this information to the European Commission. This commission, in turn, will ask the European Food Safety Authority (EFSA) for an opinion on the safety of the product, while the laboratories of the Joint Research Centre of the European Commission will be responsible for the validation of the detection method for the pertinent GMO. Within EFSA, a panel of experts on GMOs (the 'GMO Panel'), which is assisted by the EFSA staff, will prepare an opinion of the

safety of the GMO, taking into account comments that may have been received from member states' authorities regarding the contents of the dossier. The GMO Panel may also request additional information from the applicant if this is needed to be able to complete the opinion.

Based on the EFSA GMO Panel's opinion, which will be made publicly available, the European Commission will prepare a draft decision on the regulatory authorization of the pertinent GMO. The Commission may also solicit advice from a group of ethical experts, the European Group on Ethics, as well as start a public consultation. The draft decision will then be forwarded to the Standing Committee on the Food Chain and Animal Health, in which member states participate and have a right to vote. If no qualified majority of votes either in favour of or against authorization of the GMO can be reached, the draft decision is forwarded to the Council of Ministers, in which the member states' ministers in a specific area of policy participate (for example, agriculture or environment) and which also has the right to vote. If no qualified majority can be reached here either, the draft decision will be returned to the Commission, which then has to finalize its decision.

A similar procedure is followed for the environmental release of GMOs, which falls within the scope of Directive 2001/18/EC. Some differences compared to Regulation (EC) 1829/2003 have been noted with regard to the role of member states during the stages of the scientific assessment. This directive also contains a definition of genetic modification techniques, these being recombinant DNA, the introduction of externally prepared heritable material into an organism, or cell fusion methods that yield new, unnatural combinations of heritable material (Annex I of EU, 2001). Environmental release can be restricted to, for example, the import and processing of the GM crop and/or its derived viable products, such as maize kernels, or may also include cultivation. The specific scope of the application has to be indicated, as it is envisaged that this will cause great variation in the environmental issues at stake. For example, cultivation, in addition to the import and processing of a GM product, is likely to raise additional environmental issues, such as potential impacts on biodiversity in the field. Besides the potential environmental impact of a crop, such as invasiveness and impact on biodiversity of crops, weeds and insects, also potential impacts on human and animal health through accidental exposure have to be considered during the safety assessment. All these issues have to be considered during the environmental risk assessment for the GMO, of which Annex II of the Directive 2001/18/EC describes the principles. In addition, following their marketing approval GMOs should be subject to general surveillance for unanticipated adverse effects and case-specific monitoring for anticipated effects. Surveillance and monitoring plans for these unanticipated and anticipated effects have to be included with the application (EU, 2001). Because in certain instances, an applicant will wish to seek authorization for both the environmental release and food/feed uses of the GMO, combined

applications can be submitted as specified in Regulation (EC) 1829/2003 under the same regulation (EU, 2003a).

If authorization is granted, the GMO and its derived food and feed products entering the market will have to be labelled as GMOs, as explained in more detail in one of the following sections.

19.2.2 Regulatory context in the USA

While no specific law on GM crops, food or feed exists in the USA, their regulation falls within the existing framework of legislation. The Food and Drug Administration (FDA), for example, clarified its position on new plant varieties, including those obtained through modern biotechnology (i.e. new methods of genetic modification such as recombinant DNA and cell fusion), in a policy statement published in the official journal *Federal Register* in 1992 (FDA, 1992). According to this statement, the FDA treats GM crops and derived products in the same way as conventional food or feed under the Federal Food, Drugs, and Cosmetics Act, namely based upon these crops' new or altered characteristics. The process through which the product has been obtained, such as genetic modification, is therefore not the factor that determines whether or not the safety of a product has to be assessed, but may help an assessor to understand which issues have to be considered with regard to the safety and nutritional value of the final food or feed product (FDA, 1992).

The FDA's policy statement also mentions that substances that have been newly introduced into the GM crop or crop constituents whose levels have been intentionally altered by the modification are considered potentially injurious to health unless proven otherwise. These substances therefore are designated 'food additives' unless it can be shown that they are 'generally recognized as safe' (GRAS; FDA, 1992).[1] Food additives have to undergo rigorous testing and subsequent safety assessment by FDA before permission is given for their addition to foods and feed.

The FDA urges developers of GM crops to consult the agency during the development of the crop so that it can be ensured that neither issues relating to the safety of the crop for human animal health nor other regulatory issues remain unresolved when the crop is ready for commercialization. When the FDA considers that all these issues have been resolved, the consultation is deemed to have been completed (FDA, 1992).

Within the FDA, the Center for Food Safety and Applied Nutrition and the Center for Veterinary Medicine cooperate with each other on notifications for biotechnology-derived products. For the purpose of the

[1]This definition of a 'food additive' in the USA differs from that in the EU where it is considered to be a substance not normally consumed as food or food ingredient while being used for technological purposes, such as preservation, coloration, or sweetening of the food.

consultations by the developer of these biotechnology notifications, a Biotechnology Evaluation Team (BET) has been established within the FDA, consisting of a core team of experts from various backgrounds, which may be further assisted on an *ad hoc* basis by other experts. BET interacts with the developer that has consulted FDA on a GM crop. When, after initial consultations, the developer is considered to have collected sufficient evidence that the product would comply with food safety standards if commercialized, it can proceed to the final consultation by submitting a summary of the safety and nutritional data on the GM crop. BET may then decide to have an additional meeting with the developer before finalizing the consultation. It subsequently sends a memorandum to the developer of the GM crop, informing it whether there are any further outstanding questions, and if specific requirements apply to the GM crop, such as legal provisions for food additives and/or labelling requirements (FDA, 1997). Contrary to some other legislation, such as that of the EU, the FDA publishes neither a comprehensive scientific opinion nor a regulatory approval of the GM crop.

In the US, pesticidal substances expressed in GM crops fall within the scope of the Environmental Protection Agency's (EPA) oversight of pesticides as regulated by the Federal Insecticide Fungicide and Rodenticide Act (FIFRA). Pesticidal substances that have been introduced into plants through genetic modification are designated 'plant-incorporated protectants' (PIPs), which, besides naturally occurring substances and microorganisms that can be used as pesticides, fall under the broader category of 'biopesticides'. The EPA receives advice on the safety of PIPs from the FIFRA Scientific Advisory Panel (FIFRA SAP), which consists of experts in various fields of expertise and which can be supported on an *ad hoc* basis by members of the Food Quality Protection Act Scientific Review Board. If the expression levels of a given PIP are low and no indications of toxicity or allergenicity have emerged from its safety assessment, an exemption from the requirement of a tolerance level for residues of the pertinent PIP can be granted by the EPA, both for the GM crop and for the food and feed products derived from it. The EPA and FDA also require that before GM crops can be field-trialled (i.e. grown in a pre-commercial phase), their safety is assessed and an exemption granted (EPA, 2007).

The environmental release and interstate movement of GM crops fall within the regulatory oversight of the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS). Under the Plant Protection Act, APHIS implements measures to prevent the potential risks of a GM crop becoming a plant pest. If a GM crop is to be field-trialled, a request for a permit for these trials has to be obtained as the GM crop will be considered 'regulated' (i.e. subject to regulation) at this stage. A shorter notification procedure can be followed if the GM crop complies with a number of eligibility and performance criteria. The eligibility criteria include genetic stability of the introduced DNA, its

non-pathogenic sources, non-toxic and non-infectious properties of its expressed materials, and no likelihood of creation of new plant viruses. Performance criteria pertain to the way that plants and derived materials are handled, and the field trial is designed and performed. Based upon the experience obtained with regulated GM crops during field trials, the developer can file a petition for non-regulated status in order to enable the commercialization of the GM crop. At APHIS, permits, notifications, and petitions all fall within the oversight of the Biotechnology Regulatory Services, which also prepares the environmental assessments or environmental impact statements for the field trials[2] and petitions for non-regulated status of GM crops (USDA, 2007).

19.2.3 Regulatory context in Canada

In Canada, GM foods are considered a category of 'novel foods', together with foods that either have no history of safe use as a food or have been processed in a new way that causes major changes within the food beyond its natural variability. Novel foods are defined by the Food and Drug Regulations (part B, division 28 of Canada, 2011a), which fall under the Food and Drugs Act. A manufacturer or importer that intends to sell a novel food, or advertise its sale, is required to file a pre-market notification first.

Within the Food Directorate of Health Canada (i.e. the Canadian federal government's health department), a Novel Foods Section has been established that specifically deals with this kind of novel food notification, which can be diverse in nature. The data packages provided with the notifications should contain data on a number of issues linked to the safety and nutritional value of the novel food, including, among other aspects, history of use, nutrition, potential toxicity and allergenicity. The Novel Foods Section will disseminate this information internally to departments ('Bureaux') that have experts in the respective areas of food safety, i.e. chemical safety, microbial hazards, and nutrition. For certain other items regarding GM crops, Health Canada may consult with the Canadian Food Inspection Agency (CFIA).

Within CFIA, the Plant Biosafety Office is responsible for the issues associated with the breeding and cultivation of 'plants with novel traits', which include GM crops, as regulated by the Seeds Regulations under the Seeds Act (part V of Canada, 2011b; CFIA, 2004). The definition of 'novel traits' includes those traits that have intentionally been introduced into the heritable material of the plant and that render its use and safety not substantially equivalent to any cultivated seed of the same species. This definition is therefore broader in scope than just GM crops, and could also cover conventionally bred crops with altered characteristics or completely new

[2] Environmental assessment for field trials only if required; not for notifications.

crops. The Feed Section within CFIA deals with novel animal feeds that contain 'novel traits', including GM crops used as feed, as required by the Feed Regulations under the Feeds Act (article 4[1] of Canada, 2011c; Health Canada, 2006). Both for plants with novel traits and for novel feeds, a pre-market notification for approval of commercialization, including a data package on the safety of the product, has to be submitted by the applicant to CFIA.

Health Canada and CFIA coordinate the regulatory approval procedures for 'crops with novel traits' that are to be used as both food and animal feed.

19.3 Regulatory safety assessment

Before the first GM crops entered the market, organizations such as the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Organization for Economic Co-operation and Development (OECD) were already engaging in activities aimed at obtaining among experts an international consensus on how to approach the safety assessment of these crops. This resulted in a series of landmark publications and expert consultations, which are reviewed into more detail elsewhere (for example, Kuiper *et al.*, 2001). This striving towards international harmonization has also led to consensus on the approach for safety assessment of GM foods and culminated in the publication of guidelines for the safety assessment of GM foods, including foods derived from GM crops, GM micro-organisms, and GM animals by Codex Alimentarius starting in 2003 (and updated afterwards; WHO, 2010).

The approach recommended by the Codex Alimentarius guidelines is based on a comparative assessment of a GM versus a non-GM crop, where the latter has a history of safe use. It is realized that the safety of most of the foods and feeds that are consumed has not been tested *per se* but acceptance of their safety is based on experience with the crops in question, including experience of their processing. Moreover, food and feeds are usually complex mixtures of substances, some of which may exert beneficial effects, while some others may show adverse effects, which will depend both on the characteristics of the substance and its intake by human and animal consumers. It is therefore not recommended to test the absolute safety of a food or feed, but instead to consider whether changes in their characteristics could raise new health concerns, i.e. to test the safety of the new crop relative to a counterpart known to be safe. Differences thus identified will be at the focus of the subsequent safety assessment. For example, if an insect-resistant crop containing a Cry protein is being assessed, the difference from its conventional counterpart may turn out to be just this Cry protein, which should then be further studied for its safety for use in human food and animal feed.

A number of issues are commonly addressed during the regulatory safety assessment of GM crops. These issues include molecular characterization, comparative analysis (composition, phenotype, and agronomic characteristics), potential toxicity, potential allergenicity, horizontal gene transfer, nutritional characteristics, and unintended effects. These are further explained in more detail below, as well as in various other sources (for example, WHO, 2010; Kok *et al.*, 2008).

19.3.1 Molecular characterization

Molecular characterization of a GM crop includes describing the DNA used for its genetic modification. The source and structure of the DNA, such as the various genes present and their components (for example, promoters, introns, coding sequences and terminators), should be outlined. Also the method through which the recipient crop has been modified should be provided. Two frequently used methods are the particle bombardment method and *Agrobacterium tumefaciens*-mediated modification with 'disarmed' plasmids. The latter in its natural form is able to form tumour-like 'crown galls' in plants, which also involves the transfer of plasmids with virulence genes to the infected plant cells. Genetic engineers have thus employed the natural capability of this bacterium to transfer genetic material to plant cells while eliminating its ability to form crown galls. In the case of particle bombardment, DNA-coated particles are accelerated and 'shot' at high speed at cells or tissues in culture (e.g. in plates). Following this step, cells or tissues that have been successfully genetically modified can be selected and plants can be regenerated from them in culture, after which further selection can take place. The nature of the inserted DNA should be determined, for example through Southern blotting and/or DNA sequencing. Also information on the recipient plant's DNA flanking the insert should be provided, which can provide insight, for example, into potential disruptions of – or interactions with – endogenous genes and the potential new formation of 'fusion proteins' encoded by rearranged DNA consisting of endogenous and introduced DNA.

19.3.2 Comparative analysis

As explained above, the cornerstone of the internationally harmonized approach towards the safety assessment of GM crops is the comparison between the GM crop and a conventional counterpart with a history of safe use. This comparison usually comprises an extensive compositional analysis of the GM crop and the conventional counterpart that have been grown in locations representative of commercial practice during field trials. This compositional analysis comprises the macronutrients, micronutrients,

antinutrients, toxins and secondary metabolites that are known to be present in the pertinent crop and to have a relevant role in the nutrition and health of humans and animals consuming the crop. Differences that are identified between the GM crop and its counterpart should then be viewed in the light of natural background variability, for example based on the values obtained for commercial reference varieties of the same crop that were grown in the same field trial, or on values from literature or databases on crop composition. It can be envisaged that, for each crop species, it will be relevant to measure particular substances, but these will differ from one crop species to another. The OECD Task Force for the Safety of Novel Foods and Feeds has developed a range of consensus documents on key compositional parameters that can be analysed in new crop varieties (OECD, 2011).

Also phenotypic and agronomic characteristics are usually measured comparatively between the GM crop and its comparator in the field. These characteristics include, for example, parameters related to crop morphology, development, reproduction, stress and/or disease resistance, and yield. The outcomes of this comparison can have value both for the food and feed safety assessment (e.g. additional indications of potential unintended effects of the genetic modification) and for the environmental assessment (e.g. likelihood of the crop becoming a weed).

19.3.3 Potential toxicity

In many cases, the introduced DNA contains genes encoding proteins. The safety assessment will then consider the potential toxicity of the newly expressed proteins, as well as other compounds that have been introduced or whose levels have been altered as a result of the genetic modification (as, for example, observed in the comparative compositional analysis described above). The assessment of potential toxicity takes into account a range of factors. Data that are available on the history of toxicity of the source of the introduced gene and the newly expressed proteins, as well as other newly introduced or altered substances in the GM crop, can provide an indication of potential toxicity. Using bioinformatics, the amino acid sequence of the newly expressed protein can be compared to the sequences of known toxic proteins stored in protein databases. Similarity to a toxic protein can indicate that the newly expressed protein shares toxic features with this protein. The resistance of the newly expressed protein to digestive protein-degrading enzymes can be tested in *in vitro* test systems, such as incubations with the stomach enzyme pepsin. This may provide insight into the likelihood that the protein sustains protein degradation during digestion and thereby be able to interact with the host's gastrointestinal system and possibly also be taken up and transported to other tissues. *In vivo* toxicity tests, such as repeated-dose oral administration of the purified protein to laboratory rodents, may be warranted for

newly expressed proteins for which insufficient data and/or experience is available.

In addition to the potential toxicity of the newly expressed protein or other specific components of the GM crop, also the potential toxicity of the whole product may be considered. This may be the case if there are indications of unintended effects that may occur in the altered crop or if the crop has been modified extensively so that it is no longer similar to a known food or feed. The study that is usually done is a 90-day oral feeding study in laboratory rodents, even in the absence of a scientific rationale for such a study, such as an effect observed in the comparative analysis (i.e. applicants for regulatory approval perform these studies frequently). It has to be cautioned, however, that, contrary to testing of pure chemicals, testing of whole foods or feeds is bound by certain limitations. This is because crop-derived foods/feeds are complex mixtures of substances, which may require special care regarding the nutritional balance of diets containing the crop-derived food/feed, their bulkiness and palatability, their compatibility with diet formulation, and the variability in their composition. This limits, for example, the dose range that can be tested with whole foods/feeds derived from GM crops and their conventional counterparts.

19.3.4 Potential allergenicity

Food allergies are hypersensitive reactions of the immune system to certain food components. Various foods are responsible for most (>90%) of the allergies reported, including soy, cereals, peanuts, nuts, dairy, eggs, crustaceans, and fish. All known food allergens, which are substances capable of eliciting allergic reactions, are proteins, whereas most proteins are not known to be allergens. Besides food allergies, also allergies that occur upon exposure to allergens through other routes, such as respiration or skin contact, exist. Allergies among animals are also known to occur, such as in pet dogs and pigs.

No single test is able to completely predict the allergenicity, i.e. the potential to act as an allergen, of newly expressed proteins. A 'weight of evidence' procedure is therefore recommended, composed of different data and tests that together provide sufficient evidence to conclude on the potential allergenicity of the protein in question. The data and tests that are commonly employed and recommended by Codex Alimentarius have a number of features in common with toxicity testing as described above and include the following:

- Data on the history of allergenicity of the source of the introduced gene (for example, is the gene derived from an allergenic food?). Many of the GM crops that have been assessed contain newly expressed proteins that are encoded by genes from a source without known allergenicity, such as soil bacteria.

- Bioinformatics-supported comparisons between the amino acid sequence of the newly expressed protein and the sequences of known allergenic proteins.
- *In vitro* resistance of the newly expressed protein to protein-degrading enzymes, including the enzyme pepsin.
- Testing for binding of the newly expressed protein by sera containing IgE-antibodies from patients who are allergic to a particular allergen. This is done when indications exist for potential cross-reactivity with known allergens, for example because the new gene comes from an allergenic source or the newly expressed protein's sequence is similar to that of a known allergenic protein.

Based on these data, it can be concluded whether the newly expressed protein is likely to be allergenic, for example by cross-reacting with other allergens in patients allergic to those allergens.

Besides the newly expressed protein, also the recipient crop of the genetic modification may have to be considered in the assessment of potential allergenicity. If the crop is known to be allergenic (e.g. soy), potential changes in the intrinsic allergenicity of the host caused by the genetic modification should be checked for. This can be done by analysing the profile of allergenic proteins within the crop, for example by measuring the level of binding of crop-allergic patients' sera to extracts of the GM crop and its conventional counterpart or by analysing the identity and binding intensity of specific proteins within the extracts, such as through two-dimensional gel electrophoresis followed by immunochemical staining of the separated protein bands with sera.

19.3.5 Horizontal gene transfer

Horizontal gene transfer is the transfer of heritable genetic material other than to offspring. Among bacteria, for example, this is a phenomenon that may well occur as various mechanisms are in place for it, such as transfer of plasmids through conjugation, phage transfer and transposon activity. Gene transfer from plants to micro-organisms under natural or unforced laboratory conditions has not, however, been observed to occur in a range of studies (reviewed by EFSA, 2009). The potential impact of a horizontal gene transfer of newly introduced genes from a GM crop to micro-organisms and others has previously received particular interest because of the presence of antibiotic resistance marker genes in some GM crops. These marker genes are used in the selection during the initial stages of the genetic modification of crop plants, in order to discern the successfully modified plants from unmodified plants based on the latter's sensitivity to the antibiotic. These genes therefore do not serve a function in the final GM crop. Besides the lack of evidence for transfer of such genes from GM crops to bacteria, also other considerations have been taken into account, such as

the natural background level of resistance to the same antibiotic among microorganisms in the environment.

19.3.6 Nutritional value

Based on differences in the levels of nutrients observed in the comparative analysis of the composition of the GM crop and its conventional counterpart, certain changes in the nutritional value can be predicted. Besides changes in the levels of nutrients, also an altered bioavailability of nutrients, for example through a change in interaction with the crop's matrix, can affect the nutritional value of the crop. In particular cases, the nutritional value of the GM crop may have been intentionally modified, as a target of the genetic modification.

The role of certain nutrients in human and animal nutrition is well known and, in such cases, these data may provide relevant inputs for assessing the potential impact associated with altered levels or bioavailability of a given nutrient in a GM crop. If needed, studies with target animals can be carried out, while also laboratory rodent feeding studies, which may have been carried out to test the safety of the whole product, can provide information on the performance of the animals and hence the nutritional value of the product. A popular model used for testing nutritional value is the rapidly growing broiler chicken, while also other species, such as lactating milk cows, can be useful for this purpose. Rapidly growing broiler chickens reach their full size within six weeks so that any change in nutritional value of the feed is likely to exert an effect on the performance of these chickens. The chickens will be checked for performance criteria, including feed intake and body weight, plus other parameters, such as mortality and post-mortem carcass characteristics. Although these feeding studies are frequently provided by applicants in the frame of regulatory procedures for GM crops, they would in many cases not have been necessary given the kind of modification and the absence of effects in the compositional analysis of the GM crop and its conventional counterpart.

The issues surrounding the safety and nutritional assessment of nutritionally improved crops have previously been considered in more detail by a Task Force of the International Life Sciences Institute (Chassy *et al.*, 2004). In a follow-up publication, these authors also explore a number of case studies on nutritionally improved crops, including lysine-enhanced maize, which could have an application in animal feed (Chassy *et al.*, 2008). Also Annex 2 of the Codex Alimentarius guidelines on the safety assessment of GM crops highlights the issues that are of particular interest for nutritionally improved crops. These issues include, for example, the safe upper levels of the nutrient in question, the different forms in which the nutrient may occur, its bioavailability, and the estimation of exposure through oral intake, taking into account different consumption patterns among populations (Codex Alimentarius, 2008).

19.3.7 Unintended effects

It can be envisaged that, in addition to the intended effect of the genetic modification, also unintended effects may occur. There can be various reasons for this, for example when the insertion of the new gene takes place at a site within the host's DNA where an intrinsic gene is located so that the latter will be disrupted by the insertion. Another reason could be a broad specificity range of a newly expressed enzyme, also converting other substrates besides the target substance. The extensive compositional analysis, as well as the analysis of phenotypic and agronomic characteristics, usually serves to provide an indication as to whether unintended effects have occurred. Supplementary indications may be obtained from animal studies with the whole product, such as laboratory rodent assays for potential toxicity and target animal feeding studies for assessing the nutritional value of the GM crop. If the unintended effect is well characterized in terms of its characteristics and the substances that are affected, the safety assessment may focus on these aspects specifically. Otherwise an oral study with the whole product may be warranted, such as the 90-day rat feeding study, taking into account the practical limitations of these studies.

19.3.8 Pesticide residues

As mentioned above, a number of GM crops are herbicide-resistant and therefore potential changes in the residues and metabolites of the target herbicide in the GM plant with regard to those in conventional plants will have to be considered. This is usually done in the frame of parallel legislation on pesticides. As discussed above for the legislation in the USA, 'plant-incorporated protectants', such as Cry proteins expressed in insect-resistant GM crops, receive a separate assessment by the EPA, whilst, in other legislations, this is part of the safety assessment of the GM crop itself. Another issue that has received interest is the potential environmental impact linked with the changes in quantities of pesticides applied to GM crops as compared to conventional crops. The conclusion of Kleter *et al*. (2007) was that there is a general downward trend in quantities of pesticides used on GM crops, which also holds true for the predicted general environmental impact of pesticides applied to these crops.

19.4 Labelling, traceability and detection of genetically modified (GM) feed ingredients

Whereas international consensus exists on the approach for safety assessment of GM crops, other regulatory requirements for these crops may differ among nations. An example of this is the different regulatory requirements for labelling, traceability and detection. In the EU, for example, Regulation (EC) 1829/2003 requires that all food and feed products that either contain

or consist of GMOs be labelled. In practice, this also means that those products that may not contain any detectable DNA or proteins derived from the GMO have to be labelled as well. In parallel to this labelling requirement, Regulation (EC) 1830/2003 also specifies a traceability requirement for operators in the food and feed production chains (EU, 2003b). Throughout the production chain, the operators have to keep records of the GM products they receive and dispatch (with accompanying information for the next operator in the chain). One of the reasons for this traceability requirement is that it would be possible to make a recall if needed. In addition, this may also help identifying the correctness of GMOs being mentioned on the label when no DNA or protein can be detected in the product, such as in highly refined edible oils. Regulation (EC) 1829/2003 also stipulates that, in case the presence of a GMO is adventitious or technically unavoidable, the product need not being labelled if the level of the GMO is below 0.9% (EU, 2003a). Regulation (EC) 1829/2003 also demands that applicants propose methods for the detection and identification of GMOs, which will subsequently be validated by the Community Reference Laboratory and its network of detection laboratories, i.e. the European Network of GMO Laboratories (EU, 2003a). These methods usually comprise a specific polymerase-chain-reaction method, which, once validated, can be used to check for the presence of the pertinent GMO in foods and feeds, such as for the verification of labels.

In contrast to the EU, labelling of GM food and feed is not required in the USA unless the GM product differs to such an extent from its conventional counterpart that it can no longer be considered equivalent. If the GM crop is not different, it is argued that it will not raise specific health concerns and therefore does not require labelling (section VI of FDA, 1992).

With regard to detection methods, the Codex Alimentarius Committee on Methods of Analysis and Sampling has drafted guidance on the criteria for methods for detection of DNA and proteins that are specific for GM foods, for both qualitative and quantitative purposes. This draft guidance still needs to be developed further before adoption (Codex Alimentarius, 2010). Moreover the Codex Alimentarius Committee on Food Labelling is developing recommendations for the labelling of GM food and food ingredients, which highlight issues regarding, for example, nutritional claims for nutritionally altered GM foods, labelling of GM foods that have been significantly altered in their chemical, physical or functional characteristics, and voluntary labelling (Appendix VII of Codex Alimentarius, 2009). These recommendations are still being discussed within this committee. Another recent development at Codex Alimentarius was the inclusion of Annex 3 dealing with low-level presence of GM crop material in food into the Codex guidelines for the safety assessment of GM-crop-derived foods. This annex pertains to the presence, within imported commodities, of traces of a GM crop that has been approved for release in another country. The annex summarizes the safety requirements for such GM foods, while also

referring to a common database to be hosted by the FAO where members shall make information on the authorizations of GM crops within their nations available (Codex Alimentarius, 2008). This also highlights the need for having detection methods available for GM crops that have not been approved within a given legislation but that still may be imported from other countries growing the same crop. To this end, the GMDD database serves as an example of a user-friendly database with information on detection methods and DNA sequences information for a wide range of GM crops (Dong *et al*., 2008). Besides the need for databases on detection methods for GMOs approved worldwide, the analytical technology may also merit from systems that can detect many different GMOs in one reaction. Examples of these are multi-methods, such as microarray analyses, which are currently being developed at RIKILT – Institute of Food Safety (e.g. Prins *et al*., 2008).

19.5 Conclusions

The overview of the regulations on GM food and feed in the EU, the USA and Canada shows that there is a distinction between the so-called 'process-based' and 'product-based' approaches towards regulating these items. The EU legislation on GMOs can be viewed as an example of the 'process-based' approach, in which the regulatory approval is mandatory for each GMO, i.e. for each food and feed that has been obtained through the process of genetic modification. The American policy on GM foods and feeds is an example of the 'product-based' approach in which the altered characteristics in the end-product determine if the product requires regulatory approval for marketing or not. Despite this distinction, it appears that for GM crops, companies use the possibility of making voluntary consultations with the FDA on the safety of their product. The Canadian legislation appears to fall between both approaches, considering genetic modification to be 'novel' and therefore subject to regulations on novel foods, which also applies to conventionally bred crops showing significant alterations.

Despite the differences between regulatory approaches, the approach for safety assessments of foods has been internationally harmonized, which has culminated in the development of Codex Alimentarius guidelines (WHO, 2010). Feed, unlike food, is, however, not covered by Codex Alimentarius guidelines, although by and large the principles for food safety assessment also apply to feed. Further harmonization in the area of feed safety assessment is desirable, particularly given the role of animal feed in the international trade of commodity crops that also comprise GM varieties. In addition to this, further harmonization of traceability aspects of GMOs would prevent undesirable trade barriers in the world market for raw materials as well as processed products.

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19.7 Appendix: list of abbreviations

APHIS, Animal and Plant Health Inspection Service
BET, Biotechnology Evaluation Team
CFIA, Canadian Food Inspection Agency
EC, European Community
EFSA, European Food Safety Authority
EPA, Environmental Protection Agency
EU, European Union
FDA, Food and Drug Administration
FIFRA, Federal Insecticide Fungicide and Rodenticide Act
FIFRA SAP, FIFRA Scientific Advisory Panel
GM, genetically modified
GMO, GM organism
IgE, immunoglobulin E
PIP, plant-incorporated protectant
USA, United States of America
USDA, United States Department of Agriculture

20

Detection of genetically modified (GM) crops for control of animal feed integrity

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Abstract: This chapter discusses several key points on the detection of genetically modified (GM) crops. Since the introduction of GM crops, the market volume has increased considerably and in parallel so have the regulations concerning the use of these crops as food or feed. Regulating novel crops requires monitoring of exports and imports of GM crops. The reliability of detection assays is heavily influenced by sampling errors, extent of feed/food processing, matrix effects and associated DNA/protein extraction protocols. In the future, novel and stacked GM traits are expected to replace or complement those that have already been introduced into agricultural crops. Consequently, methodologies and legislative approaches will have to continue to evolve to ensure the continued integrity and safety of the feed and food system.

Key words: genetically modified, biotechnology, detection, immunoassay, PCR, food, feed.

20.1 Introduction

Since humans made the transition from hunter-gatherers to agriculture, crops have been subject to anthropogenic selection in an effort to improve agronomic traits and nutritional quality. For almost 80 years, diversity in agricultural crops has been promoted through indirect genetic modifications (GM) induced by exposure to radioactivity and/or chemicals. Currently, according to the FAO/IAEA over 3000 plant mutants are registered, with more than 2000 modified plants being used for food and feed production, of which 1400 are major staples (Ahloowalia *et al.*, 2004; Kharkwal and Shu, 2009). In the last three decades, advances in molecular biology have made target-oriented gene transfer across the species barrier possible. The majority of first-generation commercialized genetically modified (GM) crops have been engineered for enhanced agronomic performance through

transformation with genes encoding either herbicide tolerance, pest resistance or both (Flachowsky and Aulrich, 2001). The cultivation of GM crops has become the subject of a global controversy over their safety, trade, regulation and implications for the environment throughout all sectors of society. As a result various countries have established regulatory frameworks either to control the production of GM crops or to restrict their importation or cultivation (Ramessar, 2008). However, as is the case for the EU, these regulations frequently apply solely to crops subjected to direct genetic modification and not to those that have been modified by exposure to radiation or chemicals (EC, 2001).

In 1996, the first GM crops serving as major feedstuffs for livestock entered the North American market. These included herbicide-tolerant soybeans and canola and pest-protected corn. During the 12-year period from 1996 to 2008, the cultivated area of GM crops increased over 74-fold to 125 million hectares globally, with an estimated net economic benefit to farming of US$10,000 million in 2007 (James, 2008). Regulations concerning GM plants were established by major international organizations prior to their commercialization (Lezaun, 2006; Cantley, 2007; Hug, 2008). The policy of substantial equivalence was first introduced by the Organisation for Economic Co-operation and Development (OECD, 1993) and was adopted by both the Food and Agriculture Organization (FAO) and the World Health Organization (WHO, 2010) as the most appropriate regulatory framework (FAO/WHO, 2000). Substantial equivalence was based upon comparison of GM plants to an appropriate conventional counterpart from which the GM line was derived. Once defined plant traits had been deemed equivalent between the two lines, the novel transgenic trait became the focus of the safety assessment. Detailed information about the feeding qualities of GM plants for livestock and their nutritional evaluation have been reviewed previously (Flachowsky *et al*., 2005; Alexander *et al*., 2006). Since the late 1980s, the European Union (EU) has introduced a variety of legislative acts that are targeted specifically at regulating GM crops. By treating transgenic traits as a regulatory category, the principle of 'substantial equivalence' as a regulatory framework was essentially abandoned, but this approach remains the foundation of North American regulatory regimes (Lezaun, 2006).

20.2 Detection of genetically modified (GM) plants

> None of our 'common' foods and feeds
> were investigated nearly as intensively,
> regarding to human, animal and environmental safety,
> as were their transgenic counterpart (Flachowsky, 2007)

20.2.1 Rationale for monitoring GM crops

Technological innovations, including the development of GM crops, are likely to be viewed from different perspectives depending on a country's

geographic size, governmental framework, economic status, strength in science and technology, and sensitivity towards issues around food security and safety. According to Maslow's hierarchy (Maslow, 1987) of needs, food ranks among the top and is obviously essential for human survival. Populations that have experienced significant food shortages due to either natural or anthropogenic disasters may be conditioned to ask the question 'I am hungry, do I have something to eat?'. Conversely, nations with a secured food supply may ask the question 'I am hungry, what do I eat?'. Individuals from countries that have experienced restricted food supply may be far more concerned about food security than food safety. Consequently, regulatory approaches can also be greatly influenced by which of the two above scenarios have been experienced by a national population. Optimally, regulatory approaches will consider both food security and food safety as well as implications for trade and methods of enforcement.

Since their first commercialization, countries have chosen different approaches to regulate GM crops (Cantley, 2007). In the United States of America, GM products do not require labelling, but there is still a need to be able to detect those GM traits that may have implications for human health. For example, GM crops approved only for use in animal feed may inadvertently end up in food destined for human consumption, as occurred when StarLink® corn was used in the manufacture of tacos (Dorey, 2000; Fox, 2001). However, many GM crops are exported, raising the possibility that these materials may enter countries in which regulatory approval for their use has not been granted (Richmond, 2008).

To be imported into the EU, GM crops can only be approved by regulatory authorities after the completion of an extensive safety assessment which is harmonized through a centralized procedure (Regulation (EC) No. 1829/2003) (EC, 2003a). According to 1829/2003, all foods and feeds containing more than 0.9% threshold GM crops must be labelled. A threshold of 0.5% has been established for GM crops that have received a favourable risk assessment but have not yet been approved within the EU. Unapproved varieties are managed with zero tolerance for detectable material. This differs from previous legislation which mandated that foods containing detectable GM plant material in the form of either DNA or proteins must be labelled (EC, 2000). The thresholds allow for the adventitious or technically unavoidable presence of GM crops in foods/feeds (Waiblinger *et al.*, 2007). However, with the growing global adoption of GM crops, monitoring and enforcing of these threshold levels has become increasingly difficult (Weighardt, 2006). Products derived from livestock fed transgenic feeds including milk, meat, and eggs are exempt from EU labelling laws.

In compliance with Regulation (EC) No. 1830/2003 (EC, 2003b), and in order to help implement the labelling legislation of Regulation (EC) No. 1829/2003, any feed product containing more than the allowable threshold content of GM crop must be accompanied by proper documentation

stating the constituents present in the crop or food/feed product. A unique identifier describing the GM crop must be provided at each processing stage prior to the marketing of the food or feed product. However, for these maximum allowable levels to be imposed, validated analytical tests are needed to quantify the level of the transgenic trait in mixed feeds and food.

Protocols to monitor GM crops are available from the International Organization for Standardization (ISO, 2010) and analytical standards of GM crops are available from the Joint Research Centre (JRC), Institute for Reference Materials and Measurements (IRMM), a Directorate-General within the European Commission. Both the sampling procedure and the analytical precision of the analysis method influence the accuracy and precision of detecting transgenic traits in feed (Heinemann *et al*., 2004; Michelini *et al*., 2008). As GM plants can be produced by introducing transgenic DNA that either encodes a protein or possesses antisense properties that impede protein expression, it can be identified through the detection of either transgenic DNA or recombinant proteins or both.

20.2.2 Sampling of plant material

> The accuracy of many analytical data reports is a mirage because unwitting negligence and false cost consciousness have ensured that a sample of powder taken with cursory swiftness has been examined with costly precision.
> Kaye, Illinois Institute of Technology, 1967 (Gy, 2004c)

In 2007, corn and soybeans were the principal GM crops grown globally, with exports from producing countries of 110 million and 74 million metric tons, respectively. In the EU, this amounted to importation of 24 million tonnes of corn and 18 million tonnes of soybean in 2007 (FAO, 2010). Considering the potential amount of imported crops in relation to the amount of GM analyte, sampling can have a significant effect on the likelihood of detection. The science of sampling feed and food for GM traits has been extensively described by Gy *et al*. (2004a, b, c, d, e).

The technical and statistical aspects of the sampling procedures are critical to reliable detection of GM products in feeds. Sampling error can arise from execution of inadequate collection plans, an error that is magnified as the concentration of GM trait in the mixture decreases (Gilbert, 1999). Sampling strategies to monitor target material are described by the European Commission (EC, 2004) and ISO 24333:2009 (ISO, 2010) and further discussed in Anon. (2007). When designing guidelines, it is important to be aware of the buyer's and seller's risks with the probability of an incorrect assessment decreasing as the number and size of samples collected are increased (Whitaker *et al*., 2001). However, for a detection methodology to be adopted, the sampling strategy and analysis method need to achieve an

acceptable balance between sampling error and the cost to be adopted to attain an acceptable level of confidence in the result.

Any sampling plan should require that the random sample is large enough to represent the entire lot of analyte with a certain degree of statistical confidence. The robustness and reproducibility of the analytical method used to test for the presence of a GM trait will affect the nature of the sampling strategy required to gain confidence in the outcome (Remund *et al*., 2001). The United States Department of Agriculture's Grain Inspection, Packers, and Stockyards Administration personnel refer to the USDA Grain Inspection Handbook for guidelines for sampling of grains and oilseeds (USDA, 2010). However, sampling protocols not considering the lack of uniformity and randomness of GM material within large lots of feed and food may not be suitable for detection or quantification. This one factor likely accounts for the adventitious presence of GM products within some shipments. Paoletti *et al*. (2003) examined the effect of kernel heterogeneity on estimating the GM content of mixed lots of GM and conventional counterpart and found it became increasingly difficult as the heterogeneity of kernels in the mixture increased.

The method used to select laboratory samples can also be a significant source of error in the detection of GM feed. Typically, a field sample taken from a large bulk source such as a truck is sub-sampled and then that sample is ground to a fine powder. Depending on the analytical test to be performed, a sub-sample from this powder or the entire sample may be used for further analysis. For example, some lateral flow tests can accurately detect one GM corn kernel in 800 kernels using a 240 g test sample (Envirologix, 2010). For more sensitive methods such as polymerase chain reaction (PCR), 100–200 mg of test sample may be sufficient for analysis. With PCR a further sampling is required as only a sub-sample (typically, 100–200 ng) of the total extracted DNA is used in the analysis (Lipp *et al*., 2005). Considering the small amount of DNA that is used, the initial subsample size, as well as the number of replicate samples collected, is critical in determining the precision of the method. Hubner *et al*. (2001) developed a laboratory sampling scheme for the detection of GM corn and soybean by quantitative competitive PCR (QC-PCR) and real-time PCR (RT-PCR). Relative standard deviation of the procedures ranged from 5 to 20%. Using these procedures, a sample size of 10,000 seeds or kernels was required to detect the GM trait at a threshold value of 1% within a heterogeneous mixture. As sample weight varies with seed size, the amount of material required to get an adequate sample may vary with crop type, being less for crops with small seeds (e.g., 40 g for canola) than for those with larger seeds (e.g., 2850 g for corn). If the GM trait was homogeneously distributed throughout the sample, 3500 seeds were considered to be sufficient for adequate detection. Estimations of detection limits in relation to sample size should be applied to each crop in order to assess the level of confidence in detecting the GM trait.

20.2.3 Matrix effects

During extraction of a sample, other chemical components of the plant, feed additives or extraction reagents may be co-isolated with the targeted DNA or protein. Effects that these factors have on the analysis are termed 'matrix effects' (Stave, 1999). Frequently, impurities in a plant or DNA matrix can have adverse effects on detection, especially with DNA-based methods. Humic substances, plant polysaccharides and polyphenolics can inhibit PCR through the formation linkages with DNA (Wilson, 1997; Demeke and Jenkins, 2009; Reuter *et al*., 2009b). Hexadecyltrimethylammonium bromide (CTAB) and isopropanol are chemicals commonly used in DNA extraction that can also inhibit PCR at concentrations greater than 0.005% (w/v) and 1% (v/v), respectively (Peist *et al*., 2001). Consequently, an assessment of potential matrix effects is central to the development of any technique to identify feed or food that contains GM plants.

Foods and feeds are among the most complex matrixes for isolation and detection of protein or DNA. The complex chemical composition of plants along with the addition of preservatives (e.g., salts) or secondary processing such as grinding, heating or fermentation add to the complexity of matrix effects that may influence the quality and quantity of DNA or protein isolated (Gryson, 2009). It is important to test for these effects when standardizing procedures, as they can affect the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ are the concentrations of the least amount of analyte that can be reliably detected or quantified, respectively. Quantification of analyte should not be attempted below the LOQ (Hubner *et al*., 2001), which is typically 5–10 times the LOD (Holst-Jensen *et al*., 2003). For DNA-based detection methods, the LOD and LOQ depend on genome size (van den Eede *et al*., 2002) and the number of transgene inserts per genome, whereas for proteins these parameters are affected by the level of expression (Stave, 2002). Sensitivities of both assays are also governed by the type of matrix present and the extraction efficiency. To determine whether a matrix alters either the LOD or the LOQ, a comparison can be made between an analyte extracted from a feed sample with a known content of the GM trait and a standard (Stave, 1999). Standards can be produced through replication of plasmids that contain a gene coding for the targeted trait or traits. For protein-based techniques the GM protein can be produced in a defined bacterial or fungal expression system. Alternatively, a known amount of GM standard can be spiked into isolated DNA or protein derived from feed or food that originated from the non-GM parental plant. Differences between detection or quantification of the recombinant analyte with and without the matrix can then be determined (Stave, 2002).

20.2.4 Reference materials, analyte extraction

Validated reference materials are important for implementing detection systems and standardizing testing between laboratories. Twenty-one

certified reference materials (CRM) for GM crops have been approved by the EU (IRMM, 2010; e.g., soybeans, corn). Certified reference materials consist of samples of varying percentages of GM content (ranging from 0.14 up to 100 g/kg) as well as a reference standard comprising plasmid DNA that harbours the gene of interest. The CRM are available in powder forms and are diluted with the corresponding non-GM counterpart to create a single matrix. Ideally, this makes the CRM useful for testing GM food or feed derived from a single plant type. However, even for single matrixes, the powder CRM may not be suitable reference material for quantification of the GM trait as DNA and protein on a percent weight basis may vary among varieties and with growing conditions, contributing to errors in the analysis of unknowns (Holst-Jensen *et al*., 2003; Weighardt, 2006). Additionally, differences in particle size between reference standards and unknown samples could increase sampling errors or alter matrix affects (Prokisch *et al*., 2001). For example, DNA concentration is not homogeneous throughout a corn kernel and particles from the embryo harbour increased DNA concentrations as compared to those from the endosperm (Trifa and Zhang, 2004). In addition, the difference in particle size will likely affect the efficiency of extraction, with smaller particles providing greater surface area and better overall extraction. It has been proposed that the ideal particle size to ensure similar DNA distribution is between 10 and 100 μm (Prokisch *et al*., 2001). Particle size of the sample can also influence protein quantification. For example, the Cry9C protein is expressed at varying levels in the various tissues of the corn kernel, with proportionally greater concentrations occurring in the endosperm and hull (Diaz *et al*., 2002).

In collaboration among 40 international laboratories, an enzyme-linked immunosorbent assay (ELISA) for quantifying the concentration of MON810 corn in unknown samples was tested (Stave, 2002). The standard used for the ELISA was comprised of corn flour prepared at a commercial plant with each lab testing two unknown samples. The first set included flour of varying concentrations (w/w) prepared at the same plant as the standards. The second set was CRM produced by the IRMM, which consisted of ground whole corn. The concentrations of the flour unknowns ranged from 97 to 99% of the actual concentration. In contrast, the concentrations of the CRM were overestimated by 14–24%. The authors attributed this overestimation to the fact that the particle size of the standards (150 μm) differed from that of the CRM (35 μm), resulting in a disproportionate recovery of Cry1Ab protein from the CRM. Consequently, to optimize the precision of GM protein detection by ELISA, it is important that both the reference standards and unknown samples are of a similar particle size and extracted in the same manner.

Testing a sample composed of a single feed type represents the simplest matrix for the detection of GM ingredients as is the case for bulk, raw products. If a proper identity-preservation system is in place, then the need for testing processed feeds is reduced as it is known if GM material is

present or not (Brookes, 2002). However, regulators may wish to test processed food and feed products to ensure that manufacturers are complying with labelling laws (Stave, 2002). Should a manufactured feed or food product be composed of more than one plant type, than it is desirable to have a reference standard that is relevant to the food or feed mixture, as matrix effects are likely to differ between a single ingredient and a mixture. The impact that inhibitors have on PCR depends on the extent to which they can be removed during DNA purification, a factor that may also depend on diet composition.

Alternatively, extracted DNA or proteins in standardized solutions may serve as useful reference materials for GM feed detection and quantification. The merit of such standards must be tested against each type of feed to account for differences in matrix effects among diets. Furthermore, processing feed materials might change the overall composition of the product and/or alter the DNA and protein content and structure. Standardized solutions of the GM trait might have an advantage over CRM, as standards with a GM content of any percentage can be formulated (Pardigol *et al*., 2003). However, the fact that solution-based reference standards do not consider matrix effects is a significant drawback to their employment. This could be partially overcome if the DNA or protein in the reference standard were concentrated and spiked into matrices that lacked GM material but were similar to the unknown sample. Kuribara *et al*. (2002) implemented this approach when they synthesized two unique plasmid standards to quantify GM corn or soybean by RT-PCR. The corn plasmid contained sequences specific to five types of GM corn, the cauliflower mosaic virus 35S promoter and nopaline synthase terminator for general screening of GM plants, and the endogenous corn starch synthase IIb gene. The soybean plasmid was similar but specific to only one GM variety and contained a region of the soy lectin endogenous gene. Although diluted in purified salmon DNA instead of a plant matrix, the standards were effectively used for quantification of GM corn and soybean when they were mixed with conventional crops (Shindo *et al*., 2002). However, the use of plasmid DNA as standards is limited by accurate quantification of the plasmid due to its small size. In addition, plasmid DNA may increase the risk of laboratory contamination a factor that can lead to the detection of false-positive samples (Lipp *et al*., 2005).

The detection of GM crops is affected by the extraction method and the resulting purity of the analyte. One recent review compared 18 different methods for the extraction of DNA from GM crops (Demeke and Jenkins, 2009). These included the binding of DNA to silica gel and magnetic beads, polysaccharide and protein precipitation and selective DNA precipitation steps either with or without cetyltrimethylammonium bromide (CTAB). Extraction of DNA with CTAB has been examined and extensively modified with 18 variations of the procedure being described (Taylor and Powell, 1982). Both purity and yield of DNA varied among procedures as

determined by spectrophotometery and PCR amplification. The authors concluded that isolation of DNA was more rapid with commercial DNA extraction kits than with CTAB-based methods, and although the kits were more expensive, this added cost may be recovered through reduced labour.

20.3 Protein-based detection of biotech crops

20.3.1 Assays for qualitative and quantitative monitoring

Immunoassays are the gold standard for detecting and quantifying novel proteins in GM crops, with enzyme-linked immunosorbent assay (ELISA) and lateral flow strip tests being the most frequent of these procedures (Grothaus *et al.*, 2006). Immunoassays are based on the reaction of an antigen (AG), i.e., the transgenic protein, with a specific antibody (Ab) to form an Ag–Ab complex. The complex is coupled to a fluorophore for colorimetric detection. Both ELISA kits and flow strips are available for the detection of numerous GM crops.

Immunoassays are mainly used as a convenient and cost-effective method for screening large lots consisting of a single ingredient that has not been extensively processed. Recently, significant improvements in the procedure have included a shift from the use of polyclonal to more specific monoclonal antibodies and from laboratory-based ELISA to field-usable lateral flow strips which can be used at on- and off-loading points and at storage and processing facilities. The ability of the immunoassay approaches to quantify GM material has recently improved (Holst-Jensen, 2009). Currently, commercialized immunoassays have a limited ability to detect multiple traits simultaneously, but the application of microarrays (Ling *et al.*, 2007) or multicoloured immuno-beads coupled with flow cytometry (Fantozzi *et al.*, 2007) has shown promise in overcoming this limitation.

Lateral flow strips utilize a double antibody system to detect novel plant proteins in bulk and in some cases processed feeds. The strips contain excess antibodies that react specifically with the GM protein and are coupled to a colour reagent. Lateral flow strips are sensitive and, depending on the manufacturer, can detect as little as one GM corn kernel in 800 or as low as 0.125% GM content. Although they are qualitative, because they provide either a positive or a negative outcome, results in the field can be obtained in less than 10 minutes, enabling shipments containing GM material to be rapidly identified. However, test strips do appear to be subject to user error. A study conducted using test strips in a grain handling facility reported substantial variation in the detection of GM soybean depending on its concentration within the sample (Fagan *et al.*, 2001). When unknown samples ranged from 0% to 1% GM content, false positives were reported 6.7% of the time, whereas they were reported 22.3% of the time when the samples contained up to 10% GM content. The increased rate of false positives for the samples with up to 10% GM content was likely due to

cross-contamination during sample preparation at the grain handling facilities. In this same study, false negatives occurred 67.7% and 68.2% of the time when the actual GM content was 0.5% and 1%, respectively. However, at a content of 10% GM soybean, all GM samples were correctly identified. This study highlights the importance of training field personnel in proper sample handling in an effort to avoid inadvertent sample contamination and false positives.

In contrast to lateral flow strips, ELISA can quantitatively measure the presence of a GM trait. An international study among 38 laboratories examined ELISA applications to detect GM soybean expressing the recombinant protein CP4 EPSPS (Lipp *et al.*, 2000). The experiment was designed to determine if Roundup Ready® soybean could be detected above a threshold concentration of 2%. Test samples contained 0–2% GM soybean powder mixed with conventional soybean powder. The ELISA had a LOD of approximately 0.35% and identified samples with a GM concentration of less than 2% with a confidence level of 99%. Additionally, the assay was repeatable ($RSD_r = 7\%$) and reproducible ($RSD_R = 10\%$), with RSD_r and RSD_R being an indication of the precision within and among laboratories, respectively.

Immunochemical tests are limited in event-specific detection. An event-specific test is one that is solely suitable for a single transgenic event. Certain GM plants express the same protein. For example, Bt176, Bt11, and MON810 corn events all express the Cry1Ab protein to varying degrees. Determining the concentration of each individual crop-type within a mixed diet is therefore limited based on protein analysis. As mentioned, expression of GM proteins varies among plant tissues and can even be influenced by maturity of the plant (Stave, 2002). Therefore, quantifying the amount of GM trait by using standards formulated on a w/w basis may not always be appropriate.

20.3.2 Effect of feed processing

The accuracy and precision of immunological assays may become compromised if feeds or foods are processed or if the sample consists of an unknown complex matrix. Factors such as interaction of the specific Ab with non-target matrix proteins or interference from protein-binding compounds such as saponins or phenolics may contribute to imprecise analysis (Anklam *et al.*, 2002). Additionally, proteins are subject to denaturation during food and feed processing technologies, including the thermal treatments that are frequently used during food or feed preparation and packaging. This can alter the tertiary structure of the protein, thereby eliminating the ability of the Ab to bind to the target epitope. Diaz *et al.* (2002) reported that about 99% of the Cry9C protein in Starlink™ corn was denatured or degraded during the processing of corn. In contrast, Rogan *et al.* (1999) showed that oil extraction from Roundup Ready® soybeans did not have a significant

effect on the sensitivity of detecting the GM trait in soybean meal using either ELISA or western blot procedures, as LOD actually increasd from from 0.25% to 1.4% and from 0.25% to 1.0%, respectively. Heat-mediated losses due to denaturation of the protein can be partially overcome by using an Ab that is specific to the denatured form of the target protein (Stave, 2002). Currently, Strategic Diagnostics Inc. produces lateral flow strips that can identify a LOD of 0.9% for CP4 EPSPS protein in toasted soy meal. The company manufactures quantitative ELISA plates that will detect 0.1% of the CP4 EPSPS protein in toasted meals, flour, soy milk, and tofu.

While heat denaturation of proteins may be overcome by designing specific Ab to the denatured protein, losses in sensitivity in other forms of food or feed preservation may be unavoidable. For example, the concentrations of Cry1Ab protein in fresh pre-ensiled corn forage were 4.9 and 8.5 μg Cry1Ab/g dry matter for the early- and late-maturing varieties, respectively (Folmer *et al*., 2002). However, the amount of Cry1Ab decreased to unquantifiable levels after nine days of ensiling for the early and after four days for the late variety. Extensive degradation of proteins during fermentation likely makes immunoassay-based detection methods impractical for the quantification of GM crops in ensiled feeds.

20.4 DNA-based detection of biotech crops

> PCR is a tool that has the power to create new situations for its use and those required to use it (S. Scharf in Bartlett and Stirling, 2003)

20.4.1 Assays for qualitative and quantitative monitoring

In contrast to protein-based methods, DNA analysis can identify the same trait in different crops even though the event results in the production of the same recombinant protein. In addition, DNA-based approaches allow detection and quantification of GM plants that perhaps do not express any novel proteins, but are engineered to alter expression of a natural gene via either a repressor or an activator. Gene detection relies on the ability to extract DNA to detect and quantify the transgene within total endogenous plant DNA. Most DNA-based methods utilize PCR to amplify the target DNA billions of times over, thereby enabling the detection and quantification of extremely small quantities of the transgene. Factors critical to this approach are the specificity of the primers, the purity of template DNA, and the presence or absence of various PCR inhibitors. Presently, there are literally hundreds of different PCR protocols for the detection of GM traits in food or feed (Holst-Jensen, 2009; JRC, 2009). A comprehensive database for the detection of GM plants has been established which outlines the detection strategies, the reference materials/molecules required and the suggested primer sets (Dong *et al*., 2008).

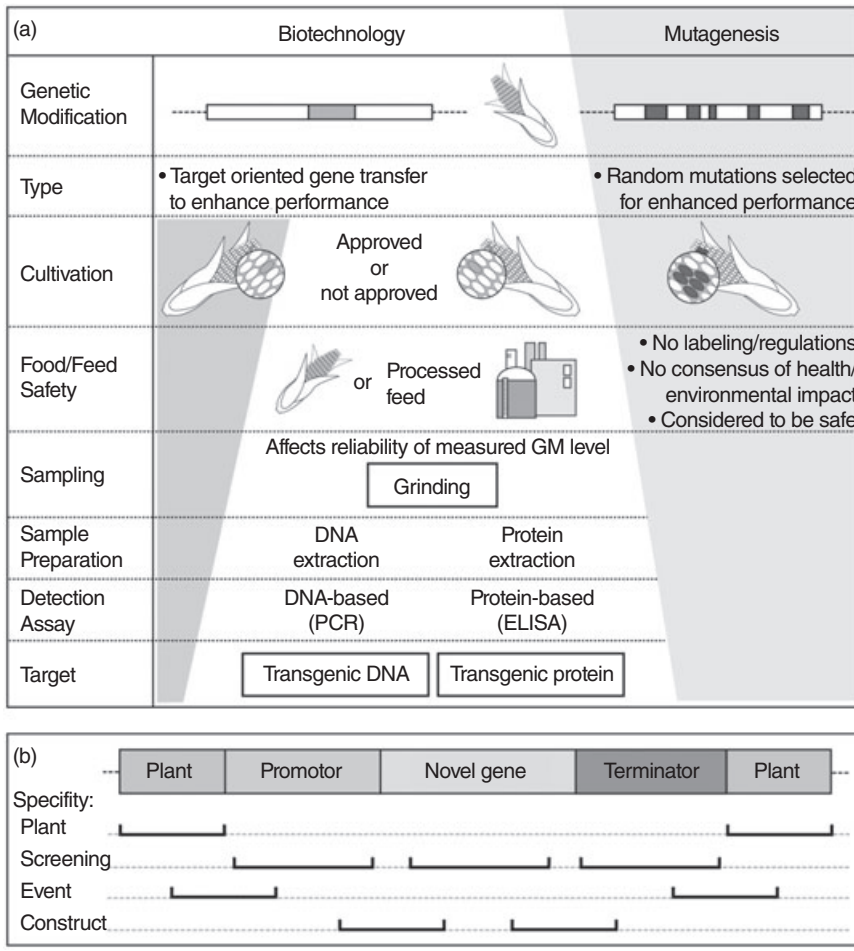


Fig. 20.1 (a) Schematic outline of the monitoring of crops genetically modified by biotechnology or mutagenesis, after cultivation with or without safety approval. Regulated crops require detection according to established international thresholds and/or labelling laws within matrixes of processed or unprocessed analytes and varying lot sizes. The reliability of target qualification and quantification is affected by matrices, the sampling and extraction protocol and the type of detection assay. Preferred detection assays are the polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA). ELISA targets the novel protein using the specificity of antibodies. PCR targets specific fragments (b) of endogenous, novel or unique cross-border DNA sequences.

There are essentially four approaches to using PCR for the detection of GM plants (Fig. 20.1(b)), with the preferred approach depending on the overall information one wishes to derive from the amplification process (Holst-Jensen *et al.*, 2003). The first approach is essentially a screening method used to detect the presence of a GM trait without necessarily

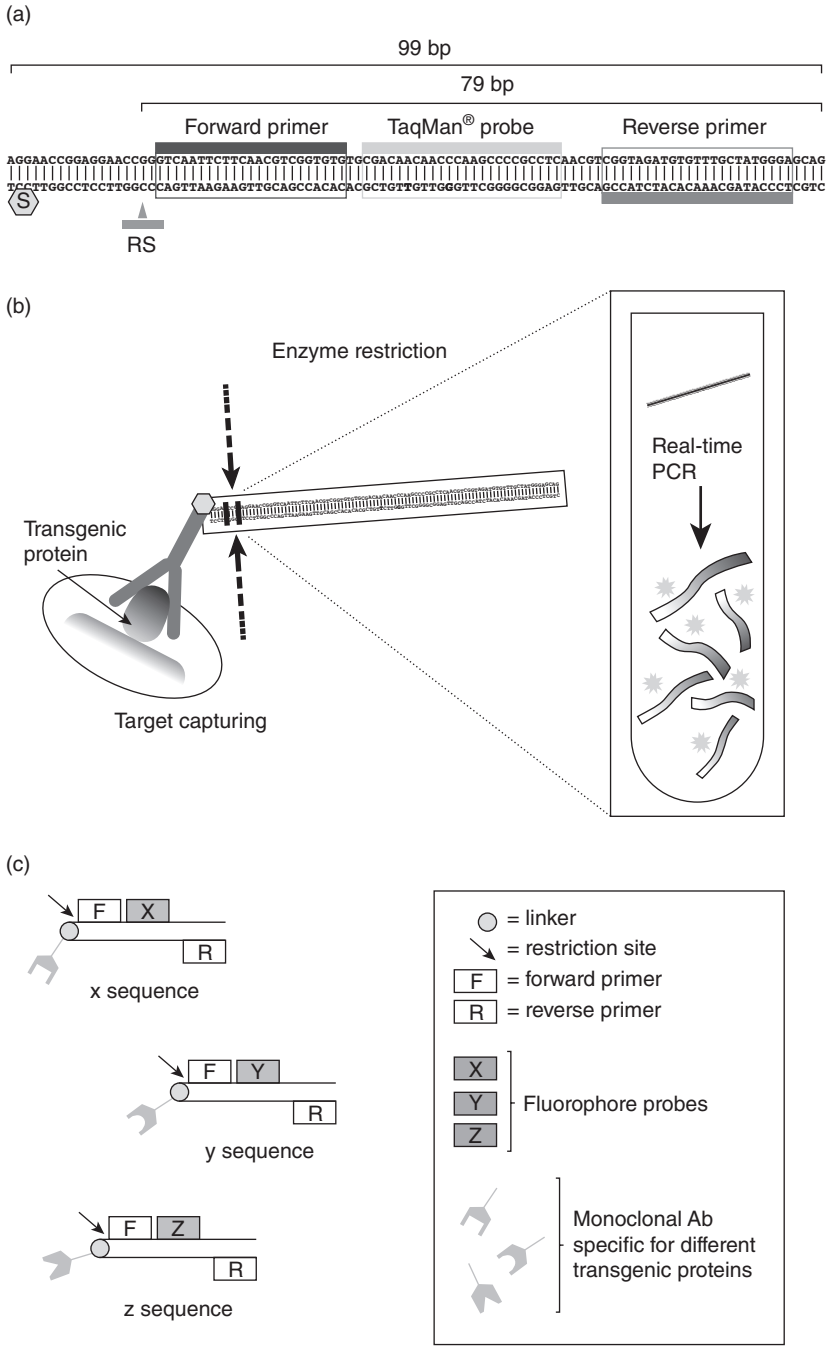
linking the trait back to the crop type from which it was derived. Such an approach is possible because many crop types are genetically modified using constructs having an identical or a very similar sequence. The majority of GM plants have been transformed with constructs containing either the Cauliflower Mosaic Virus (CaMV) 35S promoter (*P-35S*) or the *Agrobacterium tumefaciens* nopaline synthase terminator (*T-Nos*) which are used to regulate expression of the transgene. As well, the most common cloning vectors used in the past contained genes coding for resistance to ampicillin (*bla*) or neomycin/kanamycin (*nptII*), which in some cases were inserted into the plant genome during the transformation process. Consequently, primers designed for amplifying DNA sequences of the *P-35S*, *T-Nos*, *bla* or *nptII* can be used to screen for a wide range of GM crops (Vollenhofer *et al*., 1999). Such an approach is especially useful for mixed diets containing multiple GM crop types. However, it is also possible to have false positives with this approach as CaMV can be associated with infected plants (Wolf *et al*., 2000). The same result is possible for genes coding for antibiotic resistance as these genes may be harboured within bacteria that are present on the surface of the crop or present in food or feed. Additionally, a number of GM plant varieties lack the DNA sequence coding for resistance to antibiotics, as these genes are now being excised from the transformation vector prior to commercial release of the crops.

The second approach is to use PCR to specifically detect the transgene of interest (Holst-Jensen *et al*., 2003) such as the *cp4 epsps* gene, which is present in Roundup Ready® varieties of corn, soybean, and canola. Although this approach is almost conclusive in identifying the sample as transgenic, it does not necessarily provide information on the type of crop harbouring this trait. This is because multiple crop types may contain the same transgene.

The third approach is construct-specific detection and involves amplification of adjacent elements of the construct. Examples include amplification of the promoter-gene or gene-terminator regions. This approach may identify the origin of the trait through association with the commercial organization that is using a company-specific transformation system. Depending on where the primers are designed within the construct, information on the construct could allow for the transformed crop to be identified.

The fourth approach targets the junction between plant genomic and transgenic DNA, thereby enabling the GM trait to be linked specifically back to a transgenic event (Holst-Jensen *et al*., 2003). Although this approach may not be necessary for lots composed of a single type of GM crop, it does have application for the detection of GM traits in mixtures and diets. Such information may play a critical role in tracing the GM trait back to the original point-source of contamination.

Conventional PCR can be used as a qualitative method to determine the presence of GM plant DNA as well as the specific transformation event it is associated with. The technique is capable of detecting GM plants at and



below the threshold set by the European Union. For example, Jankiewicz *et al*. (1999) were able to detect GM soybeans and corn at a level of 0.1% (w/w) in their respective CRM. Serial dilution of extracted GM DNA in 1000 ng of conventional DNA showed that the theoretical LOD was 0.005% (w/w). Instead of a dilution with DNA, van Duijn *et al*. (2002) mixed a CRM with ground GM soybeans to obtain mixes of 0, 0.01, 0.1, 0.5, 1, and 2% (w/w) GM material. In this study the LOD for PCR was also found to be 0.1% (w/w).

Validation studies have been completed to assess the detection limit of PCR for GM plants using both CRM as well as processed foods. In a study involving 29 laboratories, all groups were able to identify samples that contained 2% w/w GM soybean or corn using qualitative PCR (Lipp *et al*., 1999). Two PCR assays were used to detect GM soybean, targeting either the NOS terminator or the CaMV 35S promoter. Both were providing sensitive and correct identification of transgenic soybean in a CRM containing 0.1% GM material, 94.9% and 92.8% of the time, respectively. The PCR assay for GM corn was less sensitive, detecting positive samples 84.1% of the time in samples that contained 0.1% GM material. However, all assays correctly identified samples as positive 95% of the time when the CRM contained 0.5% (w/w) GM material. Lower sensitivity with the corn assay may be attributable to the larger genome in corn as compared to soybean. In this study, there were three false positives out of 310 samples, suggesting that prudent laboratory techniques were practised. A second study involving 23 laboratories investigated the ability of PCR to detect GM corn and soybean in processed foods (Lipp *et al*., 2001). The foods (polenta, acidified soybeans, infant formula, and biscuits) were comprised of 0, 2, or 100% GM material. All foods were heated to a minimum temperature of 100°C. The same genes were targeted as in the Lipp *et al*. (1999) study, but the selected primers amplified shorter sequences of transgene: 123 bp within the CaMV 35S promoter region and 118 bp within the NOS terminator. Shorter

Fig. 20.2 Schematic representation of an iQ-RT-PCR assay for the detection of transgenic proteins. (a) A synthetic 99-bp DNA sequence with the primer location and TaqMan probe (labelled with the reporter dye (6-FAM), and at the 3′ end with the quencher dye TAMRA), the enzyme restriction site (RS), and the reactive sulphur (S) incorporated as a receptor for the linker to the transgenic protein-specific monoclonal antibody. (b) Captured protein and the antibody conjugated directly to a 99-bp DNA sequence that contains a single *Scr*FI restriction site (black bars) for release of a 79-bp DNA tail. The tail is detected after exponential amplification and quantified by real-time PCR. (c) Theoretical detection of multiple transgenic proteins using epitope-specific antibodies conjugated to DNA tails. Tails have equivalent primer recognition sites, but dissimilar sequences to enable distinctive fluorescent probes to be recognized for the detection and quantification of each unique transgenic protein. A common primer recognition site ensures similar PCR efficiencies during amplification.

sequences were selected due to the assumption that the DNA would be more fragmented as a result of heating and processing. A total of 3% of the samples containing no GM material were incorrectly identified as positive. Samples containing 2% (w/w) GM material were correctly identified 98% of the time. The study therefore showed that PCR assays are capable of detecting GM constituents even if the food is subject to extensive processing. The authors noted, however, that prudent laboratory practices must be employed to avoid false positives as a result of cross-contamination. Additional interlaboratory comparisons of GM detection using the *bar* gene and the *cp4epsps* genes have been conducted, with consistent results being obtained across laboratories Grohmann *et al*. (2009).

Screening for biotech plants is laborious and costly and will likely become more so as new lines of crops with multiple traits enter the marketplace. Multiplex PCR assays have been developed to enable the simultaneous detection of multiple traits (Dorries *et al*., 2009; Bahrdt *et al*., 2010). For qualitative PCR, the products can then be differentiated based on their molecular size. A number of researchers (Permingeat *et al*., 2002; Hernandez *et al*., 2005; Matsuoka *et al*., 2001) have designed multiplex PCR assays to screen for separate GM traits including GTS 40-3-2, Bt11, MON810, T25, and GA21. These multiplex assays could detect the GM trait in material containing as little as 0.5% (w/w) GM material mixed with conventional plant material. Furthermore, a seven-target multiplex PCR for four GM corn varieties, one variety of GM soybean along with endogenous control genes for each plant species, has been designed (Germini *et al*., 2004). The LOD of this assay was 0.25% (w/w) GM content and the assay was shown to work equally well with a variety of processed foods.

In order to assess the increasing numbers of GM crops, screening assays require efficient throughput analyses. Microarray chips consist of numerous microscopic spots of target-specific DNA oligonucleotides that can be used to hybridize DNA fragments from an analyte for detection. DNA microarrays thus allow for simultaneous identification of thousands of target sequences in a single detection assay. While microarrays have yet to be extensively used for the detection of GM plants, macroarrays containing fewer DNA sequences have been developed. Leimanis *et al*. (2008) and Bordoni *et al*. (2005) monitored target DNA fragments from five different GM crops at different concentrations. Transgenic fragments were detected after exponential PCR amplification at a detection limit of 0.5% (w/w) GM material in CRM with an accuracy above 95%. However, although microarrays have proved useful as a screening tool, their ability to simultaneously quantify multiple GM traits is limited by variation in DNA extraction as well as PCR efficiencies and/or hybridization kinetics.

Real-time PCR is the preferred method for quantifying transgenic DNA through the detection of fluorescent emissions that can be equated back to the amount of target present in the original sample. Most RT-PCR assays use fluorogenic molecules specific for the target sequence emitting a

fluorescent signal when they bind to the target. Specific RT-PCR does not require post-PCR processing such as amplicon analysis through gel electrophoresis.

For analysis of transgenic DNA by RT-PCR, a standard curve based on samples containing known amounts of GM material must be developed. There are two approaches for generating standard curves (Lipp *et al.*, 2005). The first approach utilizes known amounts of DNA either on the basis of weight or by inference of the copy number of the target gene. The amount of DNA in an unreplicated, haploid nuclear genome of an organism is known as its 1C value. An extensive database on plant C-values has been summarized by Bennett and Leitch (2005). The amount of DNA associated with a C-value can be expressed on the basis of either weight or number of nucleotide base pairs (1 pg = 980 Mbp). If the genome of the plant is known along with the number of copies of the target gene per genome, then the number of gene copies in a PCR reaction can be estimated. For example, the average 1C nuclear value for corn (*Zea mays*) is 2.97 pg (Bennett and Leitch, 2005), so assuming one transgenic construct per corn genome, construction of a standard curve encompassing 10, 100, 1000 and 10,000 copies of the transgene would require 29.7 pg, 297 pg, 2970 pg, and 29,700 pg of corn DNA respectively. Measuring the percentage of a GM plant in a feed mixture using RT-PCR would require quantification of both the transgene as well as a gene specific to the crop species. By quantitating the transgene and endogenous gene in a constant amount of DNA, along with consideration for copy number in each genome, estimating the ratio of each of the genes will enable the amount of transgenic DNA present to be predicted.

A second approach to the generation of a standard curve to quantify GM material is based upon determining differences in the threshold cycle or C_T value. In this method, the standards usually consist of equal amounts of DNA extracted from ground mixtures (w/w) containing known amounts of GM plant material. PCRs with equal amounts of DNA but differences in the C_T values between the transgene and a known endogenous gene are determined for each of the standards. The type of standard curve utilized depends on whether quantification is desired from a gene-copy-equivalent perspective or on a weight/weight basis of plant material. It is important to note that the biological variability of plant C-values needs to be considered as these values can vary; for example, the 1C value of corn ranges from 2.4 pg up to 6.3 pg (Bennett and Leitch, 2005).

Several studies have shown that RT-PCR can quantify GM material in single seed samples below the level of the EU threshold. A lower LOQ of 1 mg/g has been reported for GM rapeseed (Zeitler *et al.*, 2002) and GM soybean (Huang and Pan, 2005) and assays with upper LOQ of 1000 mg/g have been designed for GM corn (Huang and Pan, 2005) and GM soybean (Huang and Pan, 2004). One inter-laboratory comparison of RT-PCR has been completed involving 22 laboratories where the percentage of Roundup Ready® soya in soya flour was estimated using a standard curve developed

using different C_T values (Hird *et al.*, 2003). The RT-PCR assay used primers specific for the transgene as well as an endogenous gene coding for lectin. The percentage of GM soya in the samples was calculated by using a matrix-matched standard curve derived from CRM. This approach exhibited a high degree of repeatability (9.3–19.3% RSD_r) and reproducibility (20.3–33.7% RSD_R). Although RT-PCR has yet to be validated for detection of GM feeds in complex diets, it has been applied to silage (Einspanier *et al.*, 2001), ground (Novelli *et al.*, 2003) and pelleted diets (Alexander *et al.*, 2004).

20.4.2 Effect of feed processing

Using PCR, Gawienowski *et al.* (1999) documented the fate of endogenous nuclear and chloroplast rubisco genes in corn after the kernels were steeped and wet-milled. The genes could be amplified in starch, germ, coarse fibre, and wet gluten after wet-milling and steeping. However, rubisco genes were not detectable after the gluten was dried at 135°C for two hours. Target genes were detected after milling, in wet gluten and gluten fractions used for the production of feed pellets. Applied heating appeared to render target DNA undetectable. Similarly, Chiter *et al.* (2000) reported that the heating of rapeseed during oil extraction completely degraded the DNA and that a 577 bp rubisco gene was no longer amplifiable after corn was heated at 95°C for five minutes. In contrast, high molecular weight (23 kbp) DNA has been reported to be present in meal arising from solvent-extracted canola seed, even after pelleting of canola meal into a complete diet (Alexander *et al.*, 2002). The discrepancies in the above studies may be attributed to differences in the duration and degree of heat that the DNA is exposed to while being processed.

While qualitative detection of transgenic DNA after feed processing may be possible, quantitative assessment of transgenic traits may be difficult due to DNA fragmentation. Yoshimura *et al.* (2005) tested the effect of heat treatment on the quantification of transgenic and endogenous DNA in ground GM corn and soybean seeds. The samples were autoclaved in water for 0, 5, 15, 30 and 60 minutes at 110°C, with a variety of primers and probe sets being used to quantify the genes. When the size of the amplicon for the endogenous gene and transgene differed, the ratio between the two genes changed with duration of autoclaving. However, the ratio of genes increased when both amplicons were of similar size. This suggests differences in extraction and/or amplification efficiencies, but differences in the degree of degradation between transgenic and endogenous DNA cannot be dismissed. Regardless, these findings emphasize the importance of selecting an appropriately sized amplicon for RT-PCR.

The size of the target DNA sequence selected has a significant impact on the detection of GM traits in feeds with DNA that has undergone considerable degradation, such as exposure to microbial nucleases during ensiling (Hupfer *et al.*, 1999). After five days of ensiling, a 1914 bp fragment of

the transgene in Bt Corn silage could no longer be detected. In contrast, a 211 bp fragment of the same transgene could still be amplified after 100 days of ensiling, with similar findings being observed for a 226 bp fragment of a corn-specifc invertase gene. Furthermore, Aulrich *et al*. (2005) were able to detect a 194 bp fragment of the transgene *Pat* in whole plant silage and mixed corn cob silage after 200 days of ensiling. Use of small gene fragments to detect GM traits in silage appears to be an appropriate practice, but it is important to note that the degree of specificity of detection often declines with fragment size. To date, quantification of GM traits in processed feed has not been reported. As both the sample matrix and the processing technology may influence the yield and quality of DNA obtained, such validation studies are required before RT-PCR can be widely recommended for quantification of GM traits in complex, processed feeds.

20.4.3 Effects on the PCR assay

In addition to matrix effects discussed above, the reliability of target quantification during PCR can be exponentially impacted by a number of factors. Key factors are the quality and ability to amplify extracted DNA and the nature of PCR ingredients (e.g. primer, enzyme, buffer, enhancer, labelled probes) as reported by Buh Gasparic and co-workers (2010). Furthermore, processes of thermal degradation and by-products over time arising from the PCR reaction can affect DNA amplification. In addition, PCR efficiencies may differ between reference materials and the analyte, resulting in an over- or underestimation of transgenic content. Efficiencies may additionally vary between the amplification of the species-specific reference gene and the transgenic target. Cankar *et al*. (2006) evaluated the impact of these effects on the reliability of real-time PCR-based quantification. Quantification was shown to be affected both additively and logarithmically by variations in DNA extraction methods, target versus reference gene amplification as well as the degree of processing of the feed or food. Depending on calculated PCR efficiencies, transgenic contents were underestimated by as much as 4% or overestimated by as much as 30%.

20.4.4 Immuno quantitative real-time PCR

PCR is a robust method with sensitivity arising from nearly exponential amplification of the target DNA fragments. Protein assays exploit the specificity of antibodies to particular epitopes, but linear signal modulation limits their sensitivity. Overall limits of LOD by currently available immunoassay-based detection methods are a function of antibody specificity, the method of signal modulation, and/or background interference that may arise in complex biological matrices. PCR sensitivity and antibody specificity have been linked in a technique known as immuno-PCR (Sano *et al*., 1992). Immuno-PCR can enhance the LOD of a given ELISA by 100- to

10,000-fold (Niemeyer *et al*., 2005). Recently, Allen *et al*. (2006) used this procedure to detect purified *Cry1Ac* protein, illustrating the potential of this technique to detect GM crops. The immuno-PCR detected *Cry1Ac* with a degree of sensitivity that was comparable to that obtained in a gold-standard insect bioassay (Allen *et al*., 2006).

The involvement of a secondary biotin–streptavidin complex in immuno-PCR assays improves signal modulation by providing up to four binding sites, but variance in binding saturation compromises quantitative determinations. This limitation has been addressed in the development of a direct immuno-quantitative real-time PCR (iQ-RT-PCR) assay based upon direct conjugation of a target-specific antibody with a synthetic 99-bp DNA tail (Fig. 20.2(a); Reuter *et al*., 2009a). The DNA tail was engineered for dissimilarity to native sequences and included a single enzyme restriction site. This enabled subsequent quantification of restricted DNA tails using real-time PCR, while excluding contamination from natural sources. The iQ-RT-PCR assay resulted in a 1000-fold increase in sensitivity as compared to a conventional commercial ELISA assay for detection of prion proteins, with sensitivity comparable to a mouse bioassay. Furthermore, the directly-labelled conjugates used in the iQ-RT-PCR assay produced an epitope-specific signal in a par-for-par relationship to the protein of interest.

The iQ-RT-PCR technique can provide multiplex-PCR capacity through the use of multiple antibody conjugates that target multiple transgenic proteins (Fig. 20.2(b)). In this application, antibodies specific to different epitopes are linked with DNA tails that contain identical forward and reverse primer sequences, but with probe sequences associated with a different fluorophore tag for each antibody. The use of identical primers ensures uniform PCR efficiencies across epitope detections.

20.5 Conclusion

The increasing demand for agricultural products for food and feed is a consequence of the continued growth in the human population. As demand has increased, the *per capita* availability of arable land has declined, a trend that has accelerated with the additional demand for agricultural land for renewable biofuel production. Advances in biotechnology could potentially enhance crop production and contribute to satisfying the global demand for food and fuel. On the other hand, novel technologies evoke mixed reactions within societies, and occasionally legislation has been passed that restricts the adoption of GM crops. Several countries regulate GM crops through the establishment of tolerance thresholds. This approach requires that GM crops be monitored by qualitative and/or quantitative detection assays to ensure regulatory compliance. Presently, DNA- or protein-based detection assays are the preferred methods for this purpose. Technical advancements in both methods have established clear procedures for the

detection of GM crops. However, with some GM crop types cultivation has become so widespread that most lots contain GM material at an adventitious level or higher. Shipments that are GM-free command a higher price, reducing their competitiveness in the food and feed marketplace. Such a scenario raises the question that if almost all samples test positive, what is the purpose of further testing? For those GM crops that become commonplace it may be that threshold levels and regulatory restrictions will eventually be abandoned. However, new GM crops are posed to enter the marketplace, many of which alter the nutrient composition of the plant (Ruiz-López *et al*., 2009) or possess stacked traits (Taverniers *et al*., 2008). As long as crops with novel GM traits continue to be released, there will be a continued need to further refine the sensitivity, precision, repeatability and versatility of GM crop detection methods.

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21

Potential contamination issues arising from the use of biofuel and food industry by-products in animal feed

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Abstract: By-products are secondary or discarded products from manufacturing. Contamination of by-products used for feed may result in carryover to animal food products and hence have impact on either animal health or food safety. Feed by-products from bioethanol production include, for example, 'dried distillers grain' (DDG) and 'dried distillers grain with solubles' (DDGS) from generation bioethanol production, C5-molasses from generation bioethanol production and glycerol from biodiesel production. By-products from food industry may comprise discarded or downgraded food and food surplus or secondary products such as peels, pulpettes, molasses, whey, mask, oil cakes, etc. Contamination of by-products and possible impacts are presented.

Key words: biofuel by-products, food industry by-products, contaminant, feed, food safety.

21.1 Introduction

By-products in feed are a considerable source of contamination of the human food chain. Several feed contamination episodes during the past decades, e.g. dioxin contamination caused by carry-over of contaminants from feed including feed by-products, has increased the awareness of the impact of feed on animal health and food safety. Today, it is well known that feed use of contaminated by-products may cause adverse health effects in livestock animals. Contaminants and natural toxins may be present in the by-products due to a natural occurrence, as environmental contaminants, from applications of pesticides, or via introduction to the products during handling, processing, fractioning, storage or transport. Also deliberate

adulteration such as addition of melamine to feed could possibly occur. Depending on their properties, contaminants and natural toxins may be transferred from the animal to animal food products such as meat, milk, eggs or organs where the contaminants may accumulate, i.e. liver and kidney. The application of good agricultural practices or HACCP (Hazard Analysis Critical Control Points), including risk analysis on the farm and in the feed, food and biofuel industry, are important measures to prevent entrance of contaminants into the feed and food chain. A by-product may be used directly as animal feed or as a feed material, i.e. as an ingredient or as a constituent in a mixture making up a compound feed.

21.2 Potential contamination issues arising from the use of biofuel by-products in animal feed

In the future, with an expected increase in the production of biofuels, large amounts of by-products from the production may be used for feed of livestock animals. Hence, it is important to assess the impact of any hazards in by-products from biofuel production on animal welfare and food safety. In order to assess the safety of the feed products the production of biofuels is here briefly described.

21.2.1 Biofuel production and by-products for feed

Over the last decade, global biofuel production has increased fivefold and in 2008 about 68 billion litres of bioethanol and 15 billion litres of biodiesel were produced globally (OECD/IEA 2010). Most of the biofuel has been produced using first-generation technology mainly based on sugar cane and corn (IEA 2009). The introduction of more biofuels is seen as an option to reduce CO_2 emissions and, for example, the EU has set a target to use 10% renewable energy such as biofuels in the transportation sector by 2020. The transportation sector produces ~25% of global energy-related CO_2 emissions and accounts for ~50% of global oil consumption (OECD/IEA 2010).

The usage of global food resources for production of biofuels is not the optimal solution to global warming. For that reason a new second-generation technology for production of biofuel using cellulose-based non-food crops is under development which may be a dominant source of biofuel in the future.

The definitions of first-generation and second-generation biofuels are as follows (IEA Bioenergy Task 39, 2009):

> '*First generation biofuels are biofuels which are on the market in considerable amounts today. Typical 1st-generation biofuels are sugarcane ethanol, starch-based or 'corn' ethanol, biodiesel and Pure Plant Oil (PPO). The raw material*

> *for producing 1st-generation biofuels either consists of sugar, starch and oil bearing crops or animal fats that in most cases can also be used as food and feed or consists of food residues.'*
>
> *'Second generation biofuels are those biofuels produced from cellulose, hemicellulose or lignin. 2nd-generation biofuel can either be blended with petroleum-based fuels combusted in existing internal combustion engines, and distributed through existing infrastructure or is dedicated for the use in slightly adapted vehicles with internal combustion engines. Examples of 2nd-generation biofuels are cellulosic ethanol and Fischer–Tropsch fuels.'*

This categorization in generations may not be permanent; for example, some authors now denote production of biofuels from algae as third-generation biofuels.

First-generation biofuels: bioethanol production

First-generation bioethanol is produced mainly in the US and Brazil from fermentation of sugar or carbohydrate-rich raw materials, which may be derived from nearly any available crop, for example maize, sugar cane, cereals, sugar beets or molasses (IEA 2008).

First-generation production of bioethanol includes either 'wet milling' or 'dry grind processing'. 'Dry grind processing' is the most frequently used production method, but both processes may also be used in parallel. In 'dry grind processing' the dry raw material is ground and water and enzymes are added to break down the starch fraction to sugars (Fig. 21.1). Then the mixture is fermented without prior separation of bran, etc., and the ethanol is distilled. The residue, 'wet lees', is centrifuged and transferred to the drying process. The centrifuge drain (thin lees) is evaporated to 'condensed distillers solubles (CDS)' which has a syrup-like consistency, and CDS is transferred to the same drying process as the wet lees, after which the water content is reduced to 12%. Drying is done by a heating process carried out at 120–130°C, whereby the yeast cultures are destroyed. The drying is carried out either by indirect heat transfer, where the wet material passes through the piping and the heat is supplied outside the pipes, or by direct heat input, where the heat (e.g. flue gases) passes directly through the wet product. As all water is removed by drying using heat instead of, for example, drainage, heat-stable contaminants present in the wet lees or thin lees may be concentrated in the final by-products for feed.

The dried product – which is a mixture of lees and CDS – is known as DDGS ('dried distillers grains with solubles'). If the lees are dried without the addition of syrup, the product is named DDG ('dried distillers grain'). The dried products DDGS and DDG can be stored for a long time and are traded globally by the feed industry. The CDS syrup may also be disposed of directly for feed use. As an example of the yields from biorefining starch to bioethanol and protein-rich animal feed, one ton of wheat may produce 335 litres of bioethanol and roughly 250 kg protein (dry weight), 160 kg

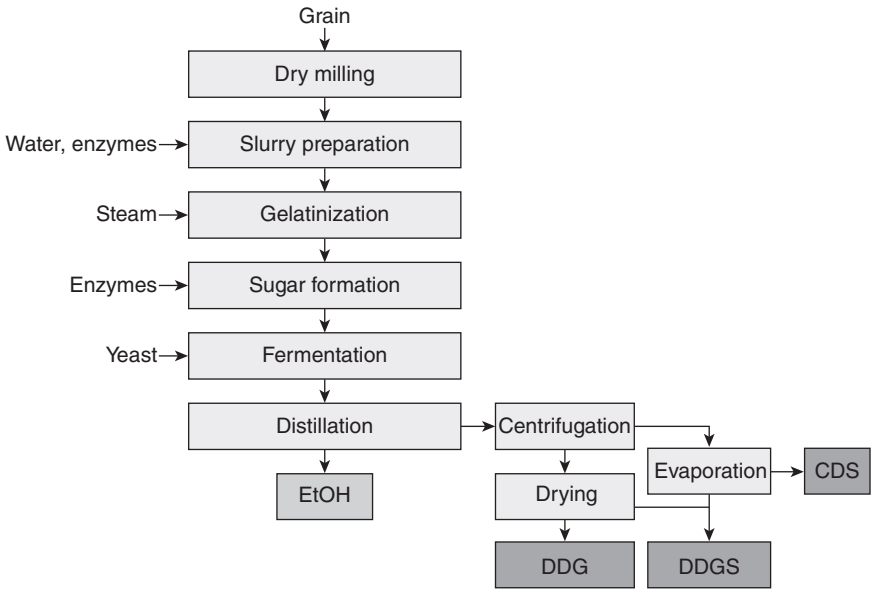


Fig. 21.1 Bioethanol production using first-generation technology and dry grind processing. The feed by-products formed are CDS ('condensed distillers solubles'), DDG ('dried distillers grain') and DDGS ('dried distillers grains with solubles').

fibres (dry weight) and 225 kg CO_2 (Danish Biofuel Holding 2010). The by-products from biorefining suitable for animal feed may in general have a final protein content of 25–35% (Ensus 2010). In 2008 1.3 million tonnes of DDGS were produced in the EU (Lywood *et al.* 2009). In the future as further DDGS feed applications are optimized, the percentage of DDGS incorporated in the feed may increase (CE Delft 2008).

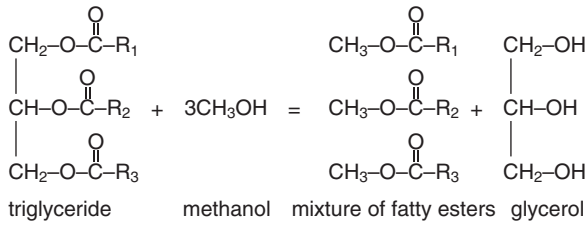
'Wet milling' is a process where the grain is steeped in a sulphuric acid solution for 1–2 days in order to separate the grain into many components. The slurry mix then passes a series of grinders for separation and the germ oil is extracted for sale while fibre gluten and starch are segregated. Wet products from biofuel production such as 'wet lees', yeast cells, etc., have a short shelf-life and for that reason they may often be used for livestock feed locally.

First-generation biofuels: biodiesel production

The raw materials used for biodiesel production are oil or fat. The main raw material for biodiesel production is crude palm oil (72%), animal fat (24%) and oil waste from cooking oil (4%) (JGSEE 2009) but all kinds of other residues may be used, for example soya beans, peanuts, cotton seed, rapeseed or any kind of seed. Most biodiesel is produced in Europe with Germany being the major producer. Compared to bioethanol the

production of biodiesel is smaller and amounted in 2007 to ~6 billion litres (2% of European diesel usage) (IEA 2008). The raw materials may be relatively expensive, as many of them also may be suitable for food.

Biodiesel is produced through a chemical process known as transesterification:



Animal and plant fats consist of triglycerides. In the triglyceride the fatty acids with the hydrocarbon chains R_1, R_2 and R_3 are esterified with the glycerol. When the triglyceride is mixed with methanol under alkaline conditions, the fatty acids are cleaved from the glycerol and esterified with the methanol to methanol esters and glycerol. Acids like sulphuric acid, hydrochloric acid or acetic acid are then used to neutralize the alkaline solution.

As the methanol esters have a lower density than the aqueous solution and phase separate, they can be fractionated and used for biodiesel products. The remaining glycerol may be used as a feed by-product. However, EU legislation prohibits the use of glycerol from the production of biodiesel based on animal products such as, for example, animal fat if the animal products themselves are not allowed to be used as feed (EU 2002, 2011). This is a consequence of the bovine spongiform encephalopathy (BSE) epidemic of the 1990s.

Today, the most widely used industrial practice for biodiesel production is the Lurgi process. In the process oil, methanol and methylate catalyst (e.g. sodium methylate) are mixed in a reactor and allowed to separate into two phases. The lighter methyl ester/oil phase is mixed with more methanol and catalyst in another reactor and gravity-separated. This second reaction optimizes the biodiesel yield and quality. The biodiesel is then washed with water to remove residues of glycerol or methanol. In addition to transesterification, an esterification technique may be used, reducing pH to less than 1 by sulphuric acid and heating at ~72ºC for two hours (EFSA 2010c).

Second-generation biofuels

Second-generation biofuels are produced from raw materials containing cellulosic biomass such as husks, leaves, stalks or wood or from dedicated energy crops such as switch grass (Table 21.1). The advantages of energy crops are that they grow easily and may be harvested several times a year. Waste products like discharged food or municipal waste may also be used as raw materials. Finally the utilization of algae biomass and their industrial

Table 21.1 Examples of raw materials used for second-generation bioethanol production

Class of residues	Production category	Examples
Primary residues	Agriculture	Straw, stover
	Forestry	Treetops, branches, stumps
	Marine	Algae
Secondary residues (production by-products)	Forestry	Bark, cellulose by-products, sawdust
	Vegetable oil production	Canola, oil palm, jatropha presscake, shells
	Sugar, first-generation ethanol	Cane, beets, bagasse, pulp, sorghum
	Crop processing	Rice, corn, coffee, cocoa (shells, husks)
Tertiary residues (after consumption residues)	Municipal waste	Furniture, demolition timber
	Food waste	Food surplus, discharged food

Source: OECD/IEA, 2010.

wastes for bioethanol production may be a sustainable approach for biofuel production (John *et al.* 2011).

There are two conversion routes of crops to second-generation biofuels (IEA Bioenergy 2009):

1. A biochemical route based on enzymatic hydrolysis of lignocellulosic material though a variety of enzymes that break the cellulosic material into sugars. In the second step of the process, these sugars are fermented into alcohol which is then distilled into ethanol.
2. A thermochemical route includes two steps of which the first is a gasification of the raw material under high temperature into a synthetic gas. This gas can then be transformed into different types of liquid or gaseous fuel, so-called synthetic fuels.

According to the International Energy Agency, biomass to liquid (BTL)-diesel and lignocellulosic bioethanol are the most promising products (OECD/IEA 2010). International enzyme-producing companies are currently launching new products for improvement of the lignocellulosic bioethanol yield. In 2010 a new technique was launched using a dual enzyme product consisting of a cellulase that converts cellulose to glucose and a hemicellulase that converts C-5 sugars to ethanol (Dong Energy 2010). Figure 21.2 shows the production of bioethanol from cellulose-based raw materials. The finely ground raw material is fed into the process

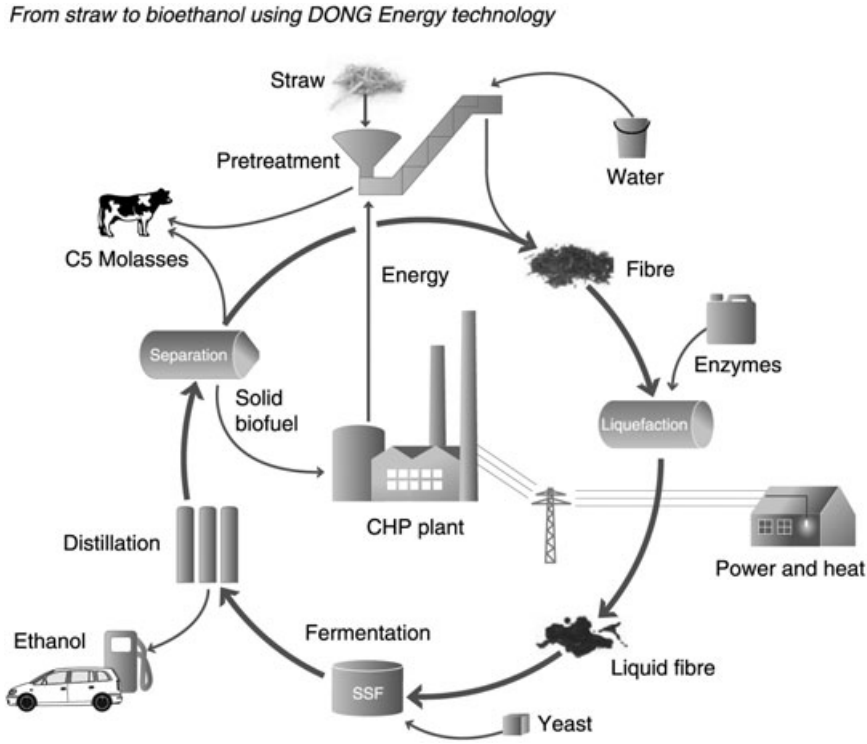


Fig. 21.2 Bioethanol production using second-generation technology and based on straw as raw material. The energy integration with a power station is shown (combined heat and power (CHP) plant). Initially steam from the power plant heats the straw and excess biofuel from the bioethanol plant is used by the power plant. The by-product C5 molasses is currently used for feed but could in the future also be used for additional biofuel production (DONG Energy 2010).

and undergoes a pure hydrothermal pre-treatment without chemicals at 180–200ºC for 5–15 min. This first step breaks down the lignin structure and fractionates the raw material into a fibre fraction for enzymatic treatment and a liquid fraction containing C5 carbohydrate salts, etc. The fibre fraction is enzymatically degraded to smaller carbohydrates, which are fermented to ethanol. The ethanol is distilled while the liquid fractions from fermentation, distillation, etc., are combined with the liquid from the pre-treatment and after evaporation C5 molasses is formed. This by-product contains a high amount of C5 sugars, which are suitable as animal feed. The output from annual use of 30,000 tonnes of wheat straw in a new Danish production plant is expected to be 11,100 tonnes of C5 molasses, 5400 m^3 of bioethanol and 8250 tonnes of lignin pellets (DONG Energy 2010).

21.2.2 Occurrences of chemical hazards in by-products from biofuel production

The main by-products from biofuel production currently used for animal feed, and therefore tested for occurrence and levels of contaminants, are CDS, DDG and DDGS from first-generation bioethanol production and glycerol from first-generation biodiesel production. Additionally some second-generation bioethanol pilot plants may deliver C5 molasses and straw molasses for use as animal feed. However, the data on contaminant levels in by-products from second-generation biofuel production are not yet available in the open literature.

The contaminants in DDG and DDGS include mycotoxins produced by moulds which historically have been known to cause adverse effects on animal and human health. Studies have shown that certain mycotoxins present in the grain for biofuel production are stable during fermentation and distillation and may concentrate up to three-fold from the raw material to the DDGS (CFIA 2009; Bothast *et al.* 1992; Wu and Munkvold 2008). The mycotoxins that may concentrate in the DDGS are aflatoxins, fumonisins, deoxynivalenol, zearalenone and ergot alkaloids. As the drying steps during first-generation bioethanol production do not include drainage, mycotoxins with relatively high water solubility are also expected to concentrate in the DDGS.

If the raw material for production of bioethanol includes food or feed crops discarded for reasons of poor quality (e.g. because of a high content of mycotoxins or other hazards), any risks regarding the chemical hazards should be considered prior to the use of residual by-products as animal feed.

The occurrence of mycotoxins in the US DDGS was tested in 235 samples collected from ethanol plants and export containers from 2006 to 2008 (Zhang *et al.* 2009). Aflatoxin and deoxynivalenol levels were all below the US FDA guidelines for animal feed, but this was not the case for fumonisin. Due to contamination with fumonisin, 10% of the samples were not recommended as feed for two species (rabbit and equids). However, the levels of fumonisin in the samples were below the US FDA guidelines for other species. Hence, in this survey where most of the bioethanol plants used corn as raw material, the majority of DDGS samples complied with the legislation.

The occurrence of contaminants in biofuel by-products was measured in samples taken in 2009 from bioethanol plants in Sweden, Germany and the Czech Republic of wheat (the raw material), wet dregs (DGS) and DDGS (Broesboel-Jensen *et al.* 2011). The samples did not belong to the same batch and for that reason it was not possible to reveal if the process concentrated the mycotoxins. The maximum content of deoxynivalenol in the raw material was 1595 μg/kg (maximum permitted level 8000 μg/kg) while the highest content in DDGS was 572 μg/kg. Deoxynivalenol is relatively thermostable up to 120°C (Hazel and Patel 2004) and the deoxynivalenol

content may increase in DDGS compared to the raw material. The mycotoxin enniatin B was found in concentrations of 1830 μg/kg in DDGS. No maximum permitted level is set in the EU for this substance. It should be noted that enniatin B is common in temperate climates. A Norwegian study showed that enniatin B was the mycotoxin with the highest prevalence (100%) in 80 wheat samples. The maximum concentration found was 5800 μg/kg (Uhlig *et al.* 2006). Ochratoxin A, which is produced during storage at humid conditions, occurred in DDGS at a maximum level of 7 μg/kg (maximum permitted level 250 μg/kg).

The DDGS samples were also analysed for pesticides and metals (Table 21.2). The only pesticide found was chlormequat. The levels of metals were generally below the maximum permitted levels; however, copper was found in amounts up to 18 mg/kg in wet dregs (DGS). In the EU copper is legislatively a food additive and the permitted levels in complete feed for pigs are 25 mg/kg and for dairy cows 15 mg/kg.

Some countries (e.g. the US and Canada) allow the use of processing additives such as antimicrobial agents to control the fermentation process by preventing bacterial growth. The Veterinary Drugs Directorate of Health Canada has assessed the potential impacts of specific antimicrobial agents and concluded that ampicillin, penicillin, streptomycin and virginiamycin used in recommended doses during the fermentation process should not result in detectable residues in the feed (CFIA 2009). However, if these by-products are to be used for feed in other countries, they should comply with current legislation which may not permit use of antibiotics.

Differences in drying method and heat intensity of DDG/DDGS batches may result in significant differences in colour quality. High heat intensity during the drying process (high temperature/long time) may scorch the feed, and several studies show a correlation between digestibility (measured by digestion of amino acids) and colour: dark-coloured DDGS has lower digestibility in both pigs and poultry (Stein *et al.* 2006). Furthermore, it is expected that dark and scorched DDGS has increased levels of, for example, acrylamide, furan or other maillard reaction products like glycotoxins. Inappropriate or error-guided direct drying processes may also lead to contamination with dioxin or PAH. Thus the colour may serve as a subjective quality criterion but it cannot replace the adequate analyses in the quality control.

The raw glycerol which is a by-product from the biodiesel production was surveyed for heavy metal residues from processing additives. Nine glycerol samples from Germany, Denmark, Brazil and the Czech Republic did not contain pesticides and the heavy metal levels were all below the maximum permitted levels (Table 21.2).

In 2010 EFSA's CONTAM panel published a scientific opinion on the abiotic risks for public and animal health of glycerine as co-product from the biodiesel production from category 1 animal by-products (ABP) and vegetable oils (EFSA 2010c). Since 2010 the glycerine by-product from

Table 21.2 Chemical contaminants in by-products

Feed by-product	Number of samples[a]	Mean (mg/kg)	Minimum (mg/kg)	Maximum (mg/kg)	Max. limit (mg/kg)
Citrus pulp	**6**				
Pesticides					
Azoxystrobin	6	0.31	0.31	0.31	1
Pyraclostrobin	6	0.05	0.03	0.10	1
Imazalil	6	0.12	<0.02	0.13	5
Thiabendazole	6	0.18	<0.02	0.37	5
Glycerol (biofuel production)	**9**				
Trace elements					
Lead (Pb)	9	1.6	<0.7	1.7	10
Cadmium (Cd)	9	0.1	<0.1	0.2	1
Chromium (Cr)	9	6.8	<1.0	12.0	
Copper (Cu)	9	3.5	<2.4	3.5	15
Manganese (Mn)	9	1.1	0.5	3.5	150
Molybdenum (Mo)	9	1.2	<1.0	1.2	
Nickel (Ni)	9	5.8	<2.2	6.4	
Zinc (Zn)	9	17	<12	17	150
DDGS, DDG (biofuel production)	**10**				
Pesticides					
Chlormequat	5	0.18	<0.01	0.34	2
Mycotoxins					
Deoxynivalenol	10	0.28	0.09	0.57	8[b]
Enniatin B	7	0.99	0.12	1.83	
Ochratoxin A	8	0.002	<0.0005	0.007	0.25[b]
Trace elements					
Lead (Pb)	3	1.5	<0.7	2.3	10
Cadmium (Cd)	8	0.19	<0.1	0.28	1
Chromium (Cr)	9	3.3	<1.0	7.9	
Copper (Cu)	10	12	7.4	18	15; 25[c]
Manganese (Mn)	10	54	27	82	150
Molybdenum (Mo)	7	1.6	<1.0	2.0	
Nickel (Ni)	4	3.2	<2.2	4.5	
Zinc (Zn)	10	63	52	80	150

[a] Number of samples with at least one analyte result above the detection limit. The sampling is not made according to current feed sampling legislation as the samples were delivered by the biofuel industries.

[b] Guidance value – Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.

[c] The 15 mg/kg applies for feed for dairy cows and 25 mg/kg for pigs, Regulation (EC) 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition.

Source: data extracted from Broesboel-Jensen *et al*., 2011.

biodiesel production is no longer categorized as a feed additive but as a feed material (EFSA 2010c).

Methanol is the major impurity of crude glycerine (glycerol) derived from biodiesel production using vegetable oils. The conclusion of the scientific opinion is, e.g., that:

> 'In commonly applied technical processes, the residual methanol concentration in the crude glycerine fraction is generally below 0.5%, below the safe content for animals. This methanol level does not represent a risk for animal health at a total inclusion level of 10% in the diet of monogastric animals and 15% crude glycerol in the diet of ruminants. The composition of crude glycerine, obtained as a co-product from biodiesel production, depends on the technical process (selection of input material) and the applied technical procedure, including the use of methanol (and in some cases of ethanol) and other catalysts (acids or bases). No data are available regarding the levels of possible contaminants of the crude glycerine obtained from biodiesel production processes using other materials than vegetable oils used for human consumption.' (EFSA 2010c).

21.3 Potential contamination issues arising from the use of food industry by-products in animal feed

Food industry by-products may be used as direct feed or as feed materials, i.e. as ingredients in a mixture making up a compound feed. During the period from 2000 to 2009 the Danish use of by-products for feed purposes, including both by-products from the food industry and other food production by-products, has roughly been at the same level of 13% (Statistics Denmark 2009). In 2009, the European use of feed by-product from the food industry was ~12% (FEFAC 2011b). The consumptions of different by-products used for feed in Denmark and Europe are listed in Table 21.3 together with the European import and consumption data for feed by-products (Statistics Denmark 2009; FEFAC 2009).

In 2008, with the aim of getting information on the usage of by-products and surplus from the production of food as feed, a questionnaire was sent to the food producers representing the largest food producers that were registered by the Danish feed authority to sell these by-products as animal feed (excluding retailers, wholesale supermarkets and producers of silage of fish waste partly used for mink feed (Broesboel-Jensen *et al.* 2011)). In total 42 of 128 producers responded – too small a feedback to get a full overview of the market, but anyhow giving insight into consumption patterns and handling of by-products. The results from the questionnaire on the by-products and surplus from food production (including discarded or downgraded foods) were divided in five categories (Fig. 21.3), the major contributors on a weight basis being by-products from the dairy industry, from category 'others' (different types of by-products belonging to the

Table 21.3 Use of by-products in agriculture (in 1000 tonnes) in Denmark and Europe

	Consumed in Denmark	Consumed in Europe	Imported to EU
	2009/2010	2009	2009
Oil-cakes and oil-meal, total	2132	40829	26195
Sunflower cakes	223		
Soya cakes	1318		
Rapeseed cakes	568		
Other oil-cakes	22		
Other vegetable feeding stuffs, total	212		
Mash, draff, yeast and molasses	170		1665
Tapioca, citrus and guar meal	42	691	1093
Meat and bone meal and fish meal etc., total	726		
Meat and bone meal	0	576	
Fish meal, fish silage and fish waste	726		562
Dairy products		1149	
Whey, milk for feeding, etc.	886		
Fat for feed	56	2187	
Beet residue and pulp	1901		515
Co-products from the food industry		17139	
DDGS			206

Source: Statistics Denmark, 2011; FEFAC, 2011a.

well-characterized class of by-products) and from beer production respectively.

In order to assess the potential risks that may be associated with the use of discarded food or by-products from the food industry, it is important to know the reason for rejection and the criteria for when a company decides to discard preprocessed or fully produced foods that were originally intended for food use. The companies were asked in the questionnaire to indicate the general reasons for discarding the products, whether physical, chemical, microbiological or for other reasons, such as, e.g., errors in production, overproduction, or exceedance of expiry date for raw materials or the final food products. The reasons most often listed by the companies (a total of 59 responses) were 'other reasons' (31) followed by 'physical' (21), then 'microbiological' (five) and finally 'chemical' (two). The by-products are mainly (29 out of 45 cases) sold locally and in many cases directly by the food-producing company.

The impacts of contaminated feed on animal health and food safety have to be assessed based on both the usage of different by-products and the

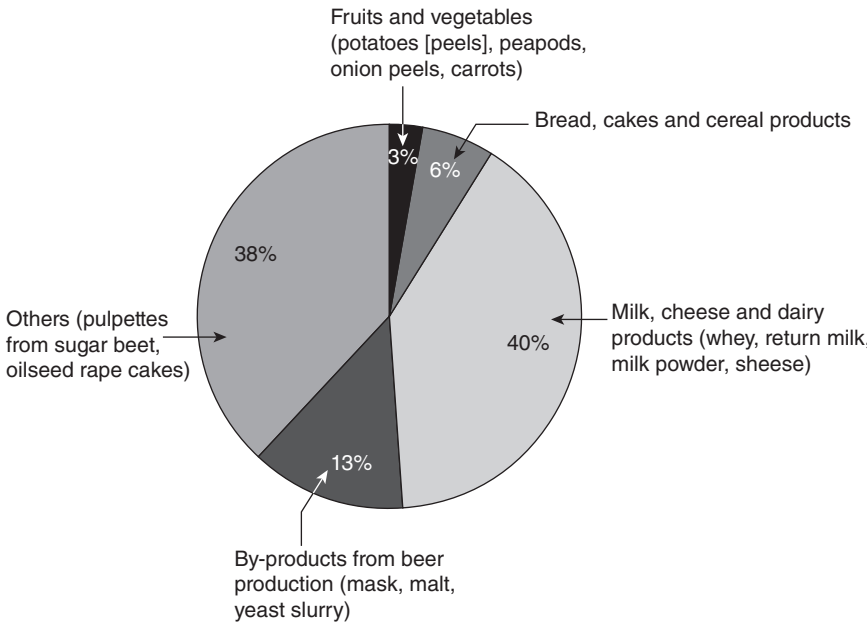


Fig. 21.3 Distribution of by-products and surplus from food industries in Denmark (data extracted from Broesboel-Jensen *et al*. 2011).

occurrence of contaminants and naturally occurring toxicants in the by-products.

The official control of chemical hazards in feed has to be both risk-based and targeted. The number of monitoring samples and the type of analyses may be revised once a year based on information from the EU rapid alert warning system, historical records of control results, and national and international specialist information. In Table 21.4 the results of the official control of feed by-products sampled by the Danish Plant Directorate from 1998 to 2008 are presented. They are summarized with respect to type of by-product and chemical compound. The chemical compounds investigated include trace elements, mycotoxins, pesticides (including chlorinated pesticides), dioxins, polychlorinated biphenyls (PCB), mustard oils, hydrogen cyanide and glycosinolates. The by-products presented are all of vegetable origin. For citrus pulp one sample contained heptachlor in a concentration exceeding the maximum residue level (MRL) and the maximum concentration of dioxin was below the MRL but >50% of MRL. The maximum concentrations of ∑DDT equalled and aldrin exceeded the MRLs. Two samples of sunflower meal naturally contained a concentration of cadmium exceeding the limit of 1 mg/kg. Furthermore a sample of rapeseed contained 4.5 times the permitted level of 100 mg/kg of volatile mustard oil,

Table 21.4 Occurrences of contaminants/residues in by-products monitored during 1996–2008

Source of by-product	Examples of by-products for feed	Contaminants	Measured maximum concentration	Number of samples*	Max. limit (MRL)
Cereals	Maize gluten, wheat-, rice- proteins, dregs	Fumonisins	2.6 mg/kg	2	60 mg/kg
Soya	Shells, toasted meal and crushed	Cd	0.13 mg/kg	11	1 mg/kg
		Pb	0.54 mg/kg	11	10 mg/kg
		Chlordan	0.01 mg/kg	1	0.02 mg/kg
		∑DDT	0.01 mg/kg	118	0.05 mg/kg
		Dioxin (TEQ)	0.09 ng/kg	1	0.5 ng/kg
		PCB (WHO TEQ)	0.04 ng/kg	1	
Citrus	Citrus pulp	Cd	0.029 mg/kg	6	1 mg/kg
		Pb	0.17 mg/kg	5	10 mg/kg
		Aldrin	0.009 mg/kg	5	0.01 mg/kg
		Heptachlor	0.016 mg/kg	2	0.01 mg/kg
		∑DDT	0.012 mg/kg	2	0.05 mg/kg
		Dioxin (TEQ)	0.28 ng/kg	11	0.5 ng/kg
		PCB (WHO TEQ)	0.015 ng/kg	3	0.35 ng/kg
		PCB	0.19 µg/kg	3	
Sugarbeet	Beet molasses, extracted, cut and pulp	Cd	0.25 mg/kg	4	1 mg/kg
		Pb	0.17 mg/kg	4	10 mg/kg
		F	67 mg/kg	14	150 mg/kg
		Dioxin (TEQ)	0.31 ng/kg	2	0.5 ng/kg
		PCB (WHO TEQ)	0.04 ng/kg	1	0.35 ng/kg
		Deoxynivalenol	0.08 µg/kg	1	5 mg/kg

Table 21.4 *Continued*

Source of by-product	Examples of by-products for feed	Contaminants	Measured maximum concentration	Number of samples*	Max. limit (MRL)
Brewery and distillery	Dregs, mash, draff, yeast and molasses	Aldrin	0.019 mg/kg	30	0.01 mg/kg
		∑DDT	0.050 mg/kg	30	0.05 mg/kg
		Aflatoxin B1	0.0005 mg/kg	18	0.02 mg/kg
		Fumonisins	1.9 mg/kg	13	60 mg/kg
Rapeseed	Cake, fatty cake and crushed	Cd	0.09 mg/kg	2	1 mg/kg
		Pb	0.3 mg/kg	2	10 mg/kg
		Dioxin	0.20 ng/kg	3	0.75 ng/kg
		PCB (WHO TEQ)	0.05 ng/kg	3	
		PCB	0.46 µg/kg	3	
		Deoxynivalenol	0.08 mg/kg	3	5 mg/kg
		Mustard oil (seed)	453 mg/kg	8	100 mg/kg
		Do. (cake, crushed)	2538 mg/kg	108	4000 mg/kg
		Glucosinolates	20 µmol	70	
		Vinylthiooxazolidon	1191 mg/kg	39	
Sunflower	Cake and crushed	Cd	1.8 mg/kg	69	1 mg/kg
		Pb	1.0 mg/kg	68	10 mg/kg
		∑DDT	0.01 mg/kg	12	0.05 mg/kg
		Dioxin	0.11 ng/kg	12	0.75 ng/kg
		Aflatoxin B1	0.0009 mg/kg	21	0.02 mg/kg

* Number of samples with at least one analyte result above the detection limit.
Source: data on by-products extracted from Broesboel-Jensen *et al.* 2011 among a total of 4237 samples of feedingstuff measured by the Danish Plant Directorate and in total 15,576 results from analyses of individual chemical compounds.

and the maximum level of volatile mustard oil in rapeseed meal was 2538 mg/kg (>50% of the permitted level at 4000 mg/kg for rapeseed meal).

21.4 The impacts on animal and human health

Risk assessment of the impacts on animal and human health from consumption of contaminated feed by-products or food contaminated from the feed may be considered on a case-by-case basis.

Risk assessment is a purely scientific process, which consist of four steps: hazard assessment, hazard characterization, exposure assessment and risk characterization (Larsen 2006). When the hazard is identified and characterized, the acceptable daily intake (ADI), tolerable daily intake (TDI) or provisional tolerable weekly intake (PTWI) values are compared to the outcome of exposure assessment in order to evaluate the impact of the assessed risk on overall food safety. The last step of risk assessment is formulation of precise advice for the subsequent risk management.

To assess carry-over of a contaminant from feed to food, the following parameters have to be considered and estimated:

1. The daily feed ration
2. The proportion of by-product in the feed
3. The period of consumption
4. Concentrations of selected contaminants in the by-product
5. The weight of the animal
6. The absorption, distribution, metabolism and excretion of selected contaminants
7. The weight of relevant animal tissues, e.g. meat, organs and milk yield.

Points 1–6 are required to assess the animal health while point 7 is needed to estimate the carry-over from feed to food.

The best way to assess the carry-over from feed to food is to determine from animal experiments the so-called transfer factors of chemicals from the animal feed to animal products. The transfer factor is expressed as the concentration of the chemical in the animal food product (mg/kg wet weight) divided by the concentration of the chemical in the animal feed (mg/kg dry weight). Leeman *et al.* (2007) has compiled data on transfer rates for a number of chemical contaminants in food products. If a study on the carry-over is not available, a conservative worst-case estimate considers that all of the contaminant consumed is transferred to the animal product, e.g. the meat.

The following two cases of mycotoxin-contaminated DDGS from biofuel production and of pesticide-contaminated citrus pulp respectively are used to illustrate the risk assessment of food industry by-products, which may have impacts on both animal and human health when included in the feed.

Table 21.5 Worst-case scenario – high inclusion of contaminated by-products in the daily feed ration for different species of livestock

	Slaughter calves	Slaughter pigs	Dairy cows
Age	7.9–12 months	147 days	–
Weight	300–450 kg	40–107 kg	600 kg
Feed ration	7.0–7.8 kg	2.09–2.57 kg	46 kg
DDGS (%)	20%	25%	4.6%
Citrus pulp (%)	22–23%	20%	12.5%

21.4.1 Case I: mycotoxins in dried distillers' grains with solubles (DDGS) from bioethanol production

This case is selected because mycotoxins are frequently found in cereal products. The mycotoxins are highly toxic and they are known to have impacts on both animal and human health. Some mycotoxins are carcinogenic. Others may have immunosuppressive effects causing other illnesses. At present, no legislation provides a basis to exclude a batch of grain contaminated with mycotoxins (and therefore discarded for feed use or for use by the food industry) from being used for bioethanol production. Therefore low-quality grain not suitable for feed or food use could be used in the production of bioethanol. As already mentioned in Section 21.2.2 it has been demonstrated that compared to the raw material (grain) some mycotoxins concentrate in the DDGS compared to the raw material during the process, as they are the remaining residues from the production and because they may resist the different processing steps: fermentation, distillation, etc.

Estimates of exposure to mycotoxins from DDGS from bioethanol production based on wheat were carried out for slaughter calves, slaughter pigs, and dairy cows. The figures for the worst-case scenarios are shown in Table 21.5. The typical inclusion of DDGS in the daily feed ration is 4–5% while a content of 20–25% was assumed as a worst case. A preliminary study on the occurrence of mycotoxins in DDGS from bioethanol production showed deoxynivalenol (DON; up to 572 μg/kg), ochratoxin A (OTA; up to 7.3 μg/kg) and enniatin B (B enn; up to 1830 μg/kg) (Broesboel-Jensen *et al.* 2011). In this worst-case scenario the calculations are carried out assuming a content of 1500 μg/kg deoxynivalenol and 20 μg/kg OTA.

For the risk assessment of DON in DDGS (1500 μg/kg, 20% inclusion rate in the daily ration) when fed to a calf weighing 350 kg (of which 210 kg (60%) is considered to provide meat for human consumption), the exposure estimate was calculated to 6.4 μg/kg body weight per day. For cattle a daily exposure at that level will not lead to safety concerns as it does not affect feed intake or weight gain – both sensitive parameters in relation to DON toxicity. Assuming that the DON in the feed is fully bioavailable to cattle and that it all ends up in the meat without elimination (worst case), the meat concentration increases by 10.7 μg/kg per day. If a person eats 5 g veal per kg body weight per day, the calf may be fed for 18 days before the

person exceeds the tolerable daily intake (TDI) of 1 μg/kg body weight. The assumption that the residues of DON are completely absorbed is unrealistic. Only a small amount (2–3%) of an ingested dose is absorbed as a parent (toxic) compound and only a minor amount enters into the meat. Furthermore the metabolism and elimination of DON are fast. Hence inclusion of 20% DDGS contaminated with DON up to a level of 1500 μg/kg in the daily ration to calves is acceptable from a toxicological point of view and will not cause safety concerns.

OTA is a mycotoxin which may impact pork production due to its nephrotoxic effect. For the risk assessment of OTA in DDGS (20 μg/kg, 25% inclusion rate in the daily ration) when fed to a pig weighing 75 kg (of which 45 kg (60%) is considered to provide meat for human consumption), the exposure estimate was calculated to be approximately 200 ng/kg body weight per day. For pigs this daily exposure to OTA is not of safety concern. Assuming that all the OTA is accumulated in the meat, the concentration increases by 285 ng/kg per day. For a person eating 5 g meat per kg body weight per day, the body burden will increase by 1.5 ng/kg body weight per day. Hence the pig could be fed the above-mentioned feed ration including 25% DDGS containing a level of 20 μg OTA per kg for 12 days before the human intake of OTA exceeds the tolerable weekly intake (TWI) of 120 ng/kg body weight. The bioavailability of OTA is closer to 60% than 100% and the OTA absorbed is mostly distributed to blood, liver and kidney and to a lesser extent to the muscles. However, the elimination of OTA in pigs is relatively slow, with a half-life of three weeks. The assumption that the concentration of OTA in the meat will increase with time is therefore reasonable. For that reason it cannot be excluded that fed rations including OTA-contaminated DDGS fed to pigs could be a risk to human health. In general, the assessment of the exposures of calves and pigs to DON and OTA consuming feed with contaminated DDGS indicates no problems regarding animal health. With regard to the human consumption of meat from cattle, pork, poultry and milk from cows fed with DDGS contaminated with DON and OTA, estimations from the above cases indicate the need for a more thorough survey/mapping of the potential concentration in meat and offal, especially from pigs that are fed OTA-contaminated DDGS.

Enniatin B (Enn B), a *Fusarium* mycotoxin which is frequently found in cereals, was also detected in DDGS. Knowledge regarding toxicity of this mycotoxin is sparse. Recently, a series of tests have shown that Enn B does not have genotoxic potential, but *in vitro* studies showed that Enn B possesses pronounced cytotoxicity (Föllmann *et al*. 2009; Behm *et al*. 2009). At present, there is no TDI for Enn B. The lack of toxicological studies on Enn B implies that the toxic potential of Enn B cannot be evaluated (the reason why Enn B has not been assessed in this case). It is important to notice that in general toxicologically poorly characterized mycotoxins such as Enn B and also newly discovered mycotoxins will not be a part of the overall risk assessment of the contamination of the food chain, nor will they be included

in the evaluation of the cumulative effects of different mycotoxins from the same feed compound.

21.4.2 Case II: pesticide residues in citrus pulp

Pesticide residues are often found in citrus. Hence pesticides in citrus pulp were selected as an example of a hazard for animal and human health. The content of pesticides in citrus pulp is evaluated based on analysis carried out by the Danish Plant Directorate (Table 21.2) (Broesboel-Jensen *et al.* 2011). The highest concentrations found were azoxystrobin 0.39 mg/kg, pyroclostrobin 0.1 mg/kg, imazalil 0.13 mg/kg and thiabendazole 0.37 mg/kg. The stobilurin pesticides azoxystrobin and pyroclostrobin were present in all six samples while the fungicides for postharvest treatment, imazalil and thiabendazol, were found in two and three samples respectively.

The risk assessment of pesticides in the citrus pulp by-product in the various animal matrices is based on the assumption that the pesticides do not metabolize in the animals. The inclusion rates of citrus pulp in the feed ration and data for the categories of livestock are shown in Table 21.5. Consumption of milk from dairy cows fed 5% citrus pulp containing the levels of pesticide residues mentioned above is considered of no safety concern for the consumer, because the exposure to these pesticide residues through the milk would be below the acceptable daily intake (ADI). This will also be the case for all other pesticides, with an ADI value below 0.007 mg/kg body weight provided the occurring residues do not exceed 0.5 mg/kg citrus pulp.

If the criterion of residual concentrations <0.5 mg/kg is maintained, there will be no health problems for consumers, using even 20% and 23% citrus pulp as feed for pigs, poultry and calves. Anyway, due to the special smell and taste of citrus pulp, the animals may reject feed rations high in citrus pulp and that may in fact limit the use of this feed material.

Still assuming that the residues are not being metabolized but end up in the milk or meat, and still maintaining the criterion of residual concentrations <0.5 mg/kg, the milk from cows fed a ration including 25% citrus pulp may exceed the ADI for some of the pesticides. However, evaluations of the specific pesticides show that they all metabolize in the animals. No pesticide residues were found in milk after feeding cattle with 0.4 mg/kg imazalil, 27 mg/kg pyraclostrobin or 20 mg/kg thiabendazole; azoxystrobin was rapidly metabolized in animals, and metabolism studies showed that transferred residues were <0.02 mg/kg (EFSA 2010a, b; FAO 2004, 2006; JMPR 1975).

21.5 Legislation and regulatory control

In the current EU legislation on feed and food it is emphasized that manufacturers are responsible for minimizing the risk of adverse effects on

human and animal health and to ensure compliance with established feed and food requirements (EU 2002). National or international legislation sets rules for handling and permissible levels of contaminants in feed including by-products intended as feed. In the absence of such legislation, standards and a code of practice on good animal feeding from the Codex Alimentarius Commission may replace it.

The Codex Alimentarius Commission (CAC) is an international body that develops standards and guidelines on food under the Joint FAO/WHO Food Standards Program. The aims are to protect the health of consumers, ensure fair trade practices in the food trade, and promote coordination of all food standards work undertaken by international governmental and non-governmental organizations. In 2004 CAC adopted and published a *Code of Practice on Good Animal Feeding* (FAO/WHO 2004). The purpose of the code is to ensure the safety of food for human consumption through adherence of good animal feeding practice at the farm level and good manufacturing practices (GMPs) during the procurement, handling, storage, processing and distribution of animal feed and feed ingredients for food-producing animals. The feed ingredients should be obtained from safe sources and be subject to risk analysis where the ingredients are derived from processes or technologies not hitherto evaluated from a food safety point of view, as may be the case for by-products, e.g. from the production of biofuels. Traceability/product trading of feed and feed ingredients including additives should enable proper record keeping for effective withdrawal of the feed if human adverse effects are identified. Feed and feed ingredients manufacturers or other relevant industry should adhere to GMP and when applicable use the HACCP (Hazard Analysis and Critical Control Points (FAO/WHO 1969)) principles to control feed-related hazards that may occur in food. They should also check that the feed meets the requirements in order to protect consumers. The Codex Alimentarius Commission sets Codex maximum residue levels and maximum permitted levels for pesticides and contaminants respectively in food; where no maximum levels for feed are set, the limit for food should apply as a minimum safety standard for feed. The Codex maximum levels are used as guidance where no other legislation is in place, e.g. in countries lacking legislation on these areas, or they can be used if national permitted levels have not been set for a particular compound. Codex standards are not legally binding, but they have significant importance in resolving disputes related to international trade.

The European legislation on animal feed provides a framework for ensuring that feed does not present any danger to human or animal health or to the environment (EU 2002; DK 2011a, b). The general Feed Safety Requirements of the European Food Law (Article 15.1) state, e.g., that

> 'Feed shall not be placed on the market or fed to any food-producing animal if it is unsafe' (EU 2002). 'By feed or feedingstuff means any substance or

> product, including additives whether processed, partially processed or unprocessed, intended to be used for oral feeding to animals' (Article 3.4). 'By unsafe is meant that the feed contains unsafe levels of for example contaminants.'

In general, the European production of biodiesel is regulated by Regulation (EC) No. 92/2005 (EU 2005b). This Regulation divides animal by-products (ABP) into three categories of risk, where Category 1 includes ABP of high-risk material and Category 3 presents ABP with a low risk to animals and humans.

Since April 2003, the European Food Safety Authority (EFSA) has been the scientific guarantor for the safety of food and feed. The EFSA's Panel on, e.g., contaminants in the food chain performs risk assessment of contamination from different sources, which also include by-products. Apart from EFSA, risk assessment is also performed at the national level.

The EU Commission has set maximum residue limits for several chemical substances in feed and food, e.g. some natural toxins, contaminants, pesticides, veterinary drugs and migrants from food contact materials. According to the European Food Law the feed companies are obliged to meet the requirements in the law and in relevant feed-related legislation.

Together the EU regulation on feed hygiene (EU 2005a) and the regulation on official controls (EU 2004) represent an EU integrated approach to ensure a high level of feed and food safety. The feed hygiene regulation is directed towards the feed companies and farms which are obliged to implement and maintain quality assurance suitable for the activities they have. All feed companies have to implement a HACCP-based system while most farmers only have to have GMP. The regulation on controls on the other hand is directed towards the competent authorities aiming to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. It demands the organizing of official controls of food and feed so as to integrate controls at all stages of production and in all sectors. The Regulation defines the European Union's obligations as regards the organization of these controls, as well as the rules which must be respected by the national authorities responsible for carrying out the official controls, including coercive measures adopted in the event of failure to comply with Community law.

Results of the national regulatory control programmes in the EU are reported to the Commission, who compile the data. However, currently publications of monitoring results of contaminants in specific feed by-products at either national or EU level are sparse.

To prevent unsafe feed from being distributed and fed to animals and to ensure efficient communication when a feed or food hazard is found in a member state, the EU Commission has established a Rapid Alert System for Food and Feed (RASFF) based on EC Regulation No. 178/2002. This

regulation states that when a member state 'has any information relating to the existence of serious direct or indirect risk to human health deriving from food or feed, this information shall immediately be notified to the Commission under the rapid alert system.' The information is then transmitted to all in the RASFF network.

The EU RASFF system (EU 2009b) may give indications of serious contaminations of by-products from the food industry or from biofuel production. From 2002 to April 2010 only one rapid alert (2007) was related to biofuel by-products, a feed-grade glycerine sample from China with a dioxin concentration of 4.98 ng/kg. However, several rapid alerts regarding compound feed have been reported, and from the reports it is not clear whether the contaminations originated from a by-product or other ingredients in the feed. Since 2000, five samples of milk by-products from Ukraine, Latvia and Lithuania contained chloramphenicol. In 2007 two soy by-products from Argentina contained the mycotoxin zearalenone in relatively high concentrations. Five samples of palm by-products contained too high levels of dioxins. Thirteen samples of fishfeed by-products contained dioxin (nine), arsenic (two) or veterinary drugs (two). A sample of a brewery by-product contained melamine (936 mg/kg) and another brewery by-product contained the unauthorized feed additive selenium in a selenium yeast product.

The notifications on dioxin included two cases of wide-scale contamination of pork through feed. In one case, pork products exported to the EU from Chile were successfully traced and recalled as a result of close cooperation between the Chilean authorities and the EU TRACES system for traceability. In the other case, within only two weeks, contaminated Irish pork was traced from raw meat to processed products. Contaminated breadcrumbs produced from bakery waste was identified as the source of dioxin due to a direct drying process, whereby combustion gases were in contact with the bread. The fuel used for heating was PCB transformer oil. The feed by-product was supplied to 10 pig and 38 beef/cattle farms. Routine monitoring by the Irish authorities detected elevated PCB concentrations in the pork, and as PCB is an indicator for dioxin the samples were analysed for dioxin. The results confirmed dioxin levels at 100 times the EU maximum level of 1 pg dioxin/g fat.

21.6 Future trends

In the future it is expected that the feed industry, the food industry and the biofuels industry will become more efficient at exploiting potential by-products of their production to be traded as feed. It seems likely that a lot of by-products will be in a form which can easily be transported and traded internationally. Opportunities to use most C5 molasses for further bioethanol production are not yet fully elucidated. For that reason it is not clear

by now to what extent C5 molasses by-products from second-generation bioethanol production will end up as feed. But other by-products from the biofuel industry may be available for feed use in the future, e.g. by-products from biofuel production based on algae (IEA Bioenergy 2009). The potential oil yields for algae are significantly higher than yields of oil seed crops and they are not in competition with regard to the availability of land. Furthermore algae have the potential to reduce the generation of greenhouse gases.

21.7 Sources of further information and advice

In 2007 a FAO/WHO expert meeting on animal feed impact on food safety highlighted the need for minimum safety criteria for safe use of feed ingredients (FAO/WHO 2008). Among others the experts expressed their concerns regarding possible contamination with mycotoxins and antibiotics of distillers' grain with solubles (DGS) and dried distillers' grains with solubles (DDGS), by-products of the bioethanol industry.

Guidance to the regulation of biofuel by-products may be found in the Canadian Food Inspection Agency's (CFIA's) regulatory guidance on 'Ethanol distillers' grains for livestock feed' (CFIA 2009) based on existing feed regulation, where usage of processing additives as antimicrobial agents and presence of contaminants are discussed. This is based on Canadian regulation but other legislations may also apply. Furthermore RASFF may give indications of serious contaminations of feed including by-products from the food industry or from biofuel production. The system has online availability and is updated daily (EU 2010).

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22

Nanoscale feed ingredients and animal and human health

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Abstract: Nanotechnology is a twenty-first century discipline that deals with the study and engineering of manufactured materials with one dimension less than 100 nm. This chapter provides an overview of the presence of this diverse group of materials in animal feed as well as the associated safety implications. Topics covered include formal definitions, aspects of their origin, and detection methods, as well as the potential biological effects of consumption of food-products containing nanomaterials for both food-producing animals and humans. Naturally occurring and manufactured materials will be discussed, and materials specifically designed to take advantage of the unique physical properties inherent to the nanoscale will be presented. Finally, the impact of these nanomaterials will be examined, both in terms of potential for tissue residues and in the development of regulations to avoid risks to health.

Key words: nanoparticles, nanomaterials, nanotoxicology, nanotechnology, food safety.

22.1 Introduction

Nanotechnology is the twenty-first century discipline concerned with studying and engineering manufactured materials with one dimension less than 100 nm. These substances range from the discipline-defining spherical buckeyball (C_{60}) of <1 nm diameter, to polymer pharmaceuticals, and to nanowires of nanometer diameter but lengths of centimeters and longer. This chapter will overview the occurrence and safety implications of this diverse group of nanomaterials entering animal feed and fodder. The first section deals with formal definitions that place topics in the proper context. Aspects of their origin, detection and potential biological effects on food-producing animals as well as human health will be presented. This will include discussions on naturally occurring versus manufactured or engineered materials, as well as materials specifically designed to take advantage of the unique

physical properties inherent to the nanoscale. The extremely high surface area to mass ratio of nanoparticles results in their surface properties having a large influence on their biological behavior as well as the ability to detect them. One issue unique to nanomaterials is their tendency to self-aggregate and/or agglomerate with natural biomolecules, which determines their biological activity as well as the extraction processes needed to detect them. How these factors impact regulations will be briefly reviewed since this area is still developing rapidly. The goal of this chapter is to review this emerging discipline relative to the safety issues related to their use in animal feeds and human food safety after consumption of animal products containing nanostructures.

22.2 Definition of a nanoscale material

Nanomaterials are materials that have a physicochemical structure on a scale greater than atomic/molecular dimensions but less than 100 nm and that exhibit physical, chemical and/or biological characteristics associated with their nanostructure. True nanotechnology involves substances that have novel properties because of their size, and if based on natural molecules (e.g., engineered biomolecules) function differently from the way intended in nature (NRC, 2006; Linkov and Steevens, 2008; Narlika and Fu, 2010). This is the so-called 'nano-effect' where unique or enhanced physical properties, reactions or biological interactions occur below a specific particle size threshold currently estimated at <100 nm. The first highly studied particle was the C_{60} carbon fullerene buckeyball, which is a single molecular cage composed solely of carbon and hydrogen atoms. Engineered nanomaterials can exhibit a variety of unique and tunable chemical and physical properties that have applications in energy, electronics, medicine and aerospace technology.

One simple physical property of nanoparticles that is often exploited is their exceptionally large surface area to mass ratio. It is their unique characteristics that have made engineered nanoparticles central components in an array of emerging technologies, and have led to commercialized products. A good example of a unique particle is the quantum dot, a colloidal semiconductor nanocrystal, which possesses stable fluorescence and has been used in a number of biomedical applications. Similarly, carbon-based nanotubes and wires have been designed with unique properties of electrical conductivity and have become central to making exceedingly strong carbon-based materials and nanosensors. Nanosized formulations of titanium dioxide have been marketed as super-efficient sunscreen formulations. A wide range of base materials are used in these applications, ranging from carbon, silica and metal oxides to numerous polymers and composite materials. The wide literature base should be consulted for specific applications. Figure 22.1 illustrates the appearance of six different nanomaterials

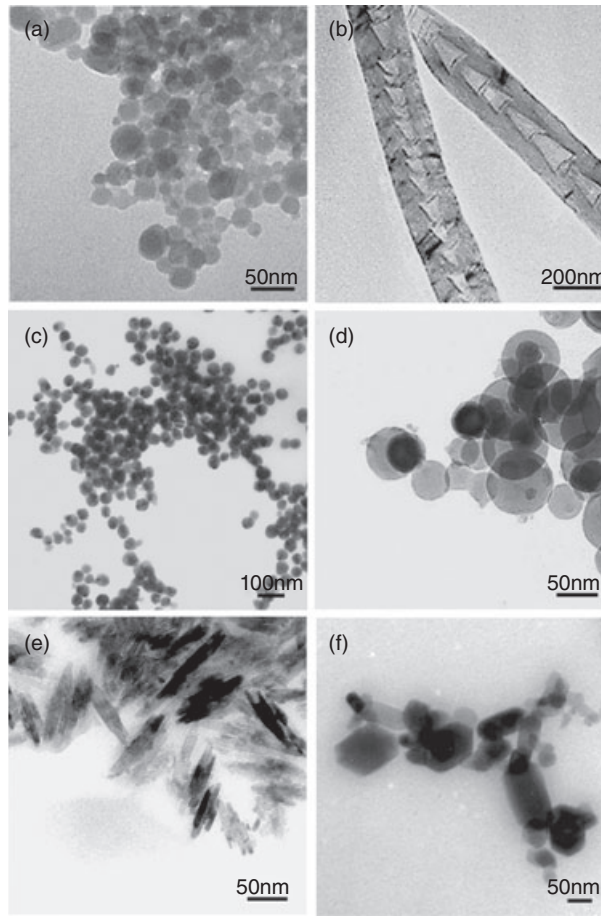

Fig. 22.1 Transmission electron micrographs of a selection of nanomaterials illustrating the large diversity in size, shape and crystalline form. A. $^{n}C_{60}$; B. multiwalled carbon nanotubes; C. 20 nm silver; D. 80 nm aluminum; E. TiO_2; F. ZnO.

to illustrate how diverse such particles can be. The water-soluble $^{n}C_{60}$, similar to particles that could occur in aqueous environments, nicely illustrates material heterogeneity (Xia *et al.*, 2010a). The multiwalled carbon nanotubes illustrate larger and more complex engineered materials, with these specific materials shown to be taken up into viable cells in culture (Monteiro-Riviere *et al.*, 2005).

In a targeted chapter such as the present one, the focus must be on general unifying concepts that could be applied to a wide range of nanomaterials. The first such concept is that size alone can be a misleading metric. A distinction must be made between a particle that just falls into a nanoscale range versus something that is truly nanoscale

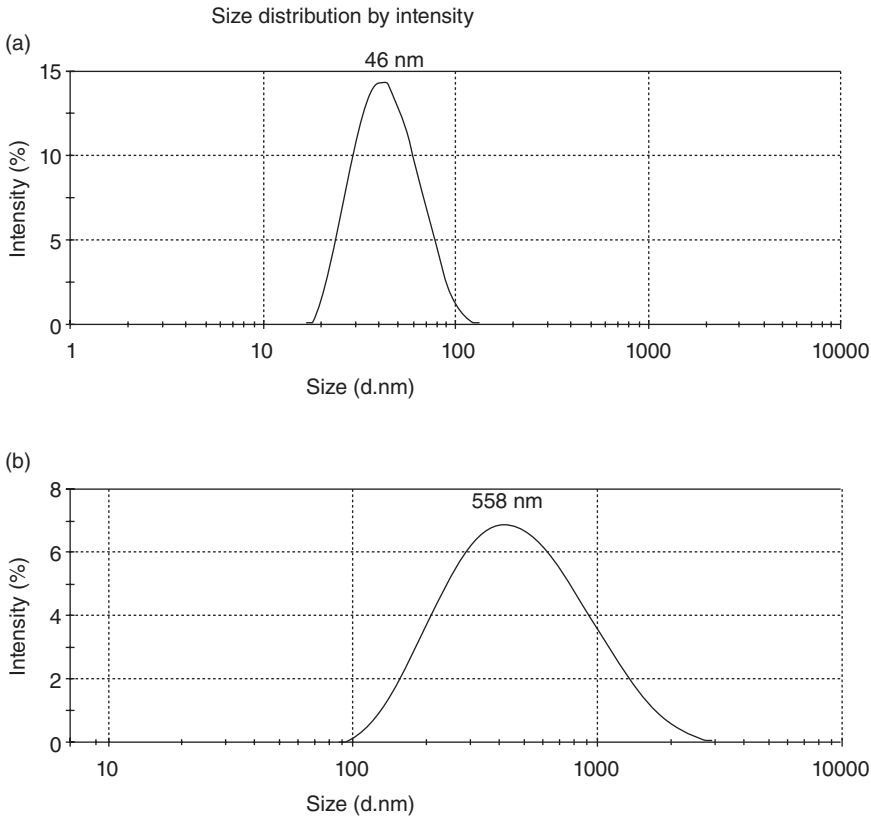

Fig. 22.2 Size distributions of model nanomaterials determined using dynamic light scattering. (a) True nanomaterial whose size distribution is centered at 46 nm and represents all nanosized material. (b) A broader population of more dispersed material that bridges nano- and micro-particles. The focus of most research related to nanomaterials is on particles with size distributions seen in (a). Although the particles in (b) are technically nanometer-sized material, most definitions would exclude them from being considered nanoparticles.

(<100 nm manufactured with unique properties). Figure 22.2 illustrates two such scenarios. In the first case (a), this is a size distribution of particles with a mean diameter of 46 nm and a distribution that is entirely within the accepted nanoparticle range of 100 nm. In contrast, case (b) is actually a microsized particle with all of its size distribution above 100 nm. The confusion often arises in that in this case, there are particles with sizes measured in nanometers; however, the consensus definition is using a cut-off of 100 nm to relate to unique physical 'nano-effects' only seen when one scale is <100 nm. When dealing with many chemical additives, this is a scenario that can be encountered but which does not properly fall under the umbrella of nanotechnology.

A similar situation occurs when true nanosized particles aggregate (bind to one another) and/or agglomerate (bind to one another and other biomolecules such as proteins) into larger-sized complexes. Because of the large surface area to mass ratio of these materials, this is a common phenomenon of nanotechnology. Although the constituent particles are nanosized, the stable complexes may not be. Such clumping can be seen in Fig 22.1 (a), (c), (d), (e) and (f). This phenomenon is often very sensitive to concentration, pH, and local ionic strength. Exposure may occur to individual nanoparticles, but when encountering biological media, agglomeration may occur and their sizes change. When this happens, particles do not exhibit the relatively unique properties associated with the nanoscale. Depending on the strength of the molecular forces involved, the process can also be reversible. It is the scenario of larger microparticles, with some spillage into the nanorange, and aggregates/agglomerates that may predominate in nanomaterial exposure in feed and fodder.

The final attribute which should be assessed is the biological implications of nanoparticles composed of inert manufactured materials versus those made from natural substances or biodegradable polymers. For example, some nanomaterials are formed from natural ingredients such as milk protein nanotubes, cellulose nanocrystals, and nanostructured liquids, and probably do not present unique concerns relative to interactions with biological systems. For so-called 'hard' engineered nanomaterials, the main concern is that after systemic absorption, there are limited mechanisms for novel inert materials to be excreted from the body. In contrast, with biodegradable polymers, natural substrates, and most nano-medicines being developed, elimination occurs, thereby avoiding long-term bioaccumulation. This issue is further discussed under health and safety in Section 22.4.

Some of the physical characteristics that have been developed to date to characterize nanomaterials are as follows:

- size and size distribution (determined using dynamic light scattering and electron microscopy)
- surface area
- surface charge
- surface chemistry
- shape
- lattice/crystal structure
- chemical composition
- aggregation status.

As this discipline develops, it is important that all such materials be fully characterized so that important attributes can be linked to biological effects. At this stage in this emerging discipline, significant research needs to define the relationship between these parameters and potential hazard and exposure. Most of the characterization metrics reflect physical structure, chemical properties, or crystalline form, attributes often determined under very

'non-biological' artificial conditions (high temperature, high vacuum, etc.). What is lacking are metrics that relate to biological interactions, such as the partition coefficient used to gauge tissue deposition for chemicals. Due to issues of colloid stability and aggregation, partition coefficients cannot be easily determined for nanoparticles. Our laboratory has recently established a metric, termed the biological surface adsorption index (BSAI), to characterize the nature of nanoparticle interactions with biological systems (Xia *et al.*, 2010b).

22.3 Origin of nanomaterials in animal feed

At this point in time, true manufactured nanomaterials have not made it into the agricultural sector. The first materials most likely to be encountered will be those microsized powders and dusts which include in their size distributions some nanoscale material (e.g. Fig. 22.2 (b)). Nanostructured liquids, polymer-based additives, and nanoscale liposomes may be encountered but should not present unique toxicological hazards to livestock nor have human food safety concerns. Their major effect may be to alter the rate and extent of absorption or biodistribution of the composite material after exposure in feed.

Based on existing projections, the following would be examples of potential areas in the future where nanomaterials would be encountered in animal feed and fodder (Kuzma and Ver Hage, 2006; NRC, 2009; Riviere, 2007):

- Nano-encapsulation of pesticide formulations to improve stability or specifically target organisms (e.g. only released in insect gut)
- Drugs formulated as block-polymers or dendrimers for increased stability and delivery
- Nano-encapsulation of vaccines
- Nanosized nutritional supplements to improve delivery
- Microbial contamination – sensors or engineered particles directed toward removal of specific organisms; antimicrobial silver nanoparticles
- Packaging material – nanocomposites to improve performance, nanosensors to detect spoilage or organic contaminants, nanoparticles containing antifungal and antimicrobial agents
- Contaminants from crops – nanomaterials used in bioremediation of farm pollutants, crop fertilizers, nanosensors used to monitor soil condition
- Natural nanomaterials – volcanic dust, anthropogenic forms such as combustion products.

As can be appreciated from the existence of relatively vague definitions of nanomaterials, it is difficult to project what may actually be encountered

and termed a nanomaterial. However, it is clear that significant research in nanotechnology is occurring in the food sector and as products and formulations develop, they may eventually enter into the animal feed cycle.

As an example, a great deal of research has been focused on applications to crop production. Carbon nanotubes have been shown to penetrate tomato seeds, and zinc oxide nanoparticles can enter the root tissue of ryegrass (Lin and Xing, 2008; Khodakovskaya *et al.*, 2009). These findings suggest that nanoformulations of essential nutrients and metal fertilizer additives may best be delivered using nanotechnology (DeRosa *et al.*, 2010). These findings also strongly suggest that nanomaterial contaminants in the environment could also end up in crops. Should this occur, then focus must be directed to the potential uptake of food animals being exposed to nanoparticles taken up into plant material components of feed or fodder.

22.4 Potential health and safety issues

The focus of this section will be to briefly overview what type of nanomaterial properties potentially could alter how a nano-formulation of a compound would require different consideration than the chemical itself, primarily from the perspective of bioavailability and subsequent biodistribution. Initial work in this field has been heavily skewed to inhalational studies and developed along the principles of particle and fiber toxicology, since this was the primary route for human occupational and environmental exposure. From this work, there have been a number of studies on biodistribution of absorbed nanomaterial and a wealth of *in vitro* studies on cellular toxicity which will not be reviewed here (Monteiro-Riviere and Tran, 2007). Since the bioavailability of nanomaterials from feed is probably limited, and data as to *in vivo* biological effects are almost nonexistent for even model materials, any discussion of potential adverse effects in humans consuming nanomaterial-contaminated meat or milk would be purely speculative.

A number of issues important to nanomaterial risk assessment depend on having knowledge of post-absorption nanomaterial biodistribution and defining which specific tissues nanomaterials become sequestered in. In addition to defining potential tissue residue issues, if they coincide with a potential target for toxicity, this knowledge is crucial to making a risk assessment for target animal safety. A unique aspect of nanoparticle biodistribution is their interaction with proteins to form a so-called 'corona'. The formation of an enveloping corona upon exposure to biological environments is a unique phenomenon of nanomaterials compared to chemical and small molecule behavior. Recent studies have suggested that it is the properties of the nanoparticle corona, when present, that often determine biological activity (Lynch and Dawson, 2008; Lynch *et al.*, 2007). This nanoparticle-protein interaction precedes its delivery to a cellular target, at

which point properties of the nanoparticle may determine the nature of any toxicity seen (e.g. oxidation of cellular target, DNA binding, etc.) (Monteiro-Riviere and Tran, 2007).

Published reviews of the field of nanoparticle pharmacokinetics and biodistribution suggest that there is sporadic literature on the pharmacokinetics of nanoparticles in laboratory animals and no studies are available in food animals (Alexis *et al.*, 2008; Li and Huang, 2008; Riviere, 2009). Most research has focused on development of nanosized drug polymers for applications in cancer chemotherapy and vaccine delivery or by using degradable nanosubstrates as carriers of small organic drugs. Specific conjugation of nanoparticles with molecular epitopes to target specific cell types has been explored. The use of nanosized formulations of traditional drugs to increase dissolution via enhanced surface area is a common theme, though not relevant to the relatively stable carbon- and metal-based manufactured nanomaterials and devices.

A great deal of the nanomedicine literature deals with biodegradable polymers and cell-specific targeting of drugs. These studies generally suggest that nanoparticle size, surface charge, and surface chemistry are important factors to consider. One reason why nanomedicine has such potential is that the larger size of nanoparticles, coupled with their tendency to form protein coronas, is generally expected to restrict tissue distribution except in so-called leaky capillary beds (pore size ≈100 nm), which include the liver, spleen, bone marrow, and tumors, making use of nanoparticles for cancer chemotherapy possible. Nanoparticles are also delivered to liver, spleen, and bone marrow because of interaction with opsonin proteins as well as antibodies and complement, which allow macrophages to engulf and sequester them. Neutral nanoparticles tend to avoid this interaction. Smaller sized nanoparticles (5–50 nm) that bind to other plasma proteins may penetrate other tissue beds and avoid this fate. Anionic quantum dots have been shown to directly enter endothelial cells in phagocytic-poor tissues both *in vivo* and *ex vivo* (Praetner *et al.*, 2010; Lee *et al.*, 2007). Recall that particulate lipoproteins, characterized by different densities (e.g. low and high density cholesterol carriers), are essentially nanoparticles that circulate in the body and are taken up into cells by specific endocytic pathways, providing a transport mechanism for nanoparticle uptake (Zhang and Monteiro-Riviere, 2009). These studies suggest that should nanomaterials be absorbed into the body after oral exposure in the diet, nanoparticles will be present in tissues and thus require consideration relative to potential residue exposure in edible products from food animals.

The limited literature of the pharmacokinetics of carbon-based material including C_{60} fullerenes and carbon nanotubes is primarily focused on therapeutic applications. A classical pharmacokinetic study was conducted in Sprague–Dawley rats using a water-soluble C_{60} derivative with antiviral properties (Rajagopalan *et al.*, 1996). Terminal half-life was approximately seven hours, volume of distribution was 2 L/kg consistent with extensive

distribution to the liver and spleen, and there was no evidence of urinary excretion. These C_{60} were 99% protein bound in plasma, illustrating the protein corona concept. Yamago *et al.* (1995) studied a ^{14}C-labeled lipophilic yet water-soluble C_{60} after IV and oral administration, with very little oral absorption detected. After IV administration, only 5% of the compound was excreted, all by the fecal route, with most of the label retained in the liver after 30 hours. In a recent study of ^{14}C-labeled C_{60} injected into pregnant rats, C_{60} accumulated in the liver but also crossed the placenta and was transmitted to offspring via the dam's milk and subsequently absorbed systemically (Sumner *et al.*, 2010). This has implications to residues in milk should a similar phenomenon occur in dairy cows or goats. Local aggregation and agglomeration of distributed nanoparticles, precipitated by local tissue pH and osmolarity, could also result in pockets of tissue accumulation.

There are systemic disposition studies reported using inherently fluorescent quantum dots derivatized for medical imaging. Similar to carbon materials reviewed above, they accumulate in the liver and spleen (Akerman *et al.*, 2002). Imaging studies in mice clearly show that quantum dot surface coatings alter their disposition and pharmacokinetic properties (Ballou *et al.*, 2004). These coatings determined *in vivo* tissue localization, with retention of some quantum dots occurring up to four months. Our group has published a physiologically based pharmacokinetic model based on reported quantum dots studies in rats (Lee *et al.*, 2009). Quantum dots had extensive distribution to tissues including the liver and kidney; however, minimal excretion occurred from the body, demonstrating bioaccumulation concerns. Choi *et al.* (2007) showed small QD with a hydrodynamic radius less than 5.5 nm could be excreted by the kidney, zwitterionic and neutral coatings reduced plasma protein corona formation, and larger sizes were not excreted. Finally, pharmacokinetic studies have been conducted with nano-dimension drug block-copolymers and dendrimers. As described above for other nanomaterials, the physical–chemical properties of the drug and polymer carrier affect disposition and circulating half-life (Kabanov *et al.*, 2002).

All these mechanisms of tissue deposition have toxicological relevance for nanomaterials. Should significant nanomaterial be absorbed systemically into a food-producing animal after exposure in feed, there would be a potential for tissue residues to occur primarily in the liver and kidney, both target organs for biodistribution due to uptake by reticuloendothelial cells. This would be accentuated for those nanoparticles not excreted from the animal. As seen in the rodent studies reviewed above, transfer to milk may also be possible. Recently, a body of research has defined the ability of orally dosed nanoparticle proteins and vaccines to be systemically bioavailable (des Rieux *et al.*, 2006), a finding consistent with the C_{60} rat studies mentioned above. Titanium dioxide nanoparticles (25 and 80 nm groups), often used as a white pigment in food products, were orally dosed to mice

and absorption was compared to microsized-particles (155 nm). Nanoscale material preferentially accumulated in the liver, spleen, lung and kidneys and induced adverse effects in the liver (Wang *et al.*, 2007). Zinc oxide nanoparticles were also shown to be bioavailable to snails after environmental exposure (Croteau *et al.*, 2011), raising the concern of inadvertent dietary exposure from environmental contamination of fodder. However, there is no direct research on nanomaterial exposure in the complex matrix of animal feed used in commercial livestock systems.

Finally, there are other potential effects of a nanomaterial contaminant being present in animal feed. For example, C_{60} fullerenes-contaminated feed would act like a 'super activated charcoal' and potentially bind other contaminants present in the feed (e.g. pesticides) or alternatively bind nutritional additives or drugs. If this bond were relatively weak, these nanoparticles could function as delivery agents for these toxins or drugs into the animal. This is the basis of a number of nanomedicines being developed.

22.5 How to differentiate between nanomaterials and organic chemicals during detection

Now that the reader has a better understanding of the definition and potential effects of nanoparticles, the question is how nanomaterial-contaminated feed and fodder, or animal tissues obtained from exposed animals, could be detected. What makes their detection different from that of any other chemical or biomolecular contaminant?

The major differences relate to sampling, extraction and analytical methodologies. Because of the tendency of nanoparticles to aggregate, distribution of material in any matrix or organ may not be uniform. Attempts to solubilize tissues and extract material using solvents commonly employed in organic chemical analysis likewise may cause aggregation if the solvent is completely evaporated, forcing aggregation. Subsequent resolubilization may not occur. This issue was addressed in designing extraction methods for chromatographic analysis of C_{60} in blood (Xia *et al.*, 2006). Binding of certain nanomaterials to sampling vials can occur.

Detection is a function of the chemical nature of the underlying material. For nanosized formulations of feed additives or drugs, the assay used to detect the chemical must be appropriate. However, derivatization of some materials (e.g. carbon fullerenes, nanotubes) may alter chromatographic absorbance. Additionally as discussed earlier, the presence of a nanomaterial contaminant may bind other feed components and prevent their detection. Another interaction is when the presence of a nanomaterial in test media alters the spectrophotometric properties of chromophores used as detectors within *in vitro* toxicology screens, a phenomenon not seen with chemical contaminants (Monteiro-Riviere *et al.*, 2009). A similar situation

could occur with chemical reactants used to detect specific materials. These issues, which are very nanoparticle specific, must be addressed early in development stages. The standard research approach to study tissue localization of nanomaterials *in vivo* has been electron microscopy, an accurate technique but one not amenable to screening.

22.6 Regulatory status

The regulatory status of nanomaterials is not well defined across applications and regulatory jurisdictions. Lack of consistent definitions applicable across multiple product areas delays regulations from being made which in turn hampers realistic risk assessments from being developed (NRC, 2009). Many existing nanoparticles are natural and many so-called nanomaterials are really not 'nano' at all. Definition and characterization become crucial.

Introduction of new technologies into food production is fraught with potential difficulties and public anxiety, as is evidenced in the debates on regulation of genetically modified organisms and food irradiation. In July 2010, the European Parliament proposed suspension of sale of food containing ingredients derived from nanotechnology. The United Kingdom has recommended that products be tracked and research specific to applications in food be pursued (Anon., 2010). In the United States, the National Nanotechnology Initiative has specifically been established to attempt to coordinate activities across all components of the federal government, from research and funding perspectives. As definitions become more focused for specific applications (e.g. powders that have nanosized constituents versus novel engineered nanomaterials), agencies such as the Food and Drug Administration responsible for regulating nanomedicines should develop guidelines to define areas of concern. The Environmental Protection Agency and the US Department of Agriculture have likewise addressed resources to define potential hazards. There is little doubt that guidelines and regulations will be issued and then revised as relevant knowledge becomes available and products enter commerce.

22.7 Future trends

As can be appreciated from the tone of this chapter, there is minimal information currently available on the presence or behavior of nanomaterials in animal feed or fodder. This is typical of an emerging discipline such as nanotechnology. The studies completed to date suggest that some materials may work their way into animal feed and fodder, potentially as contaminants or as functional additives or nanobased sensor or packaging material. The specific properties of the nanomaterial may determine its absorption profile and subsequent biodistribution in the livestock species consuming

it. Except for potential tissue residue concerns, we do not have data to complete a reasonable risk assessment. However, there is great activity in developing novel applications in microbial and chemical contaminant detection using nanosensors, packaging, and crop applications such as fertilizers, as well as nutrient supplement, vaccine and drug delivery. These materials will find applications in animal agriculture, and when this does occur, developments in detection and our knowledge of biological effects will no doubt also become more advanced.

22.8 Sources of further information and advice

Nanotechnology is a rapidly developing field and new discoveries occur on a daily basis. Major research journals such as *Nature Nanotechnology*, *Nano Letters*, *Small*, and *Nanotoxicology*, as well as review journals including *Nano Today* and *Wiley Interdisciplinary Reviews of Nanomedicines and Nanotechnology*, are potential sources of information. Discipline-oriented journals should also be scanned, since the majority of applied nanomaterial research is covered in this fashion. Government and non-government organization websites are always useful sources. This author has found the following helpful: www.nano.gov, cordis.europa.eu/nanotechnology, www.nano.org.uk, www.nanowerk.com, and www.safenano.org. A particularly useful site which tabulates nanomaterials in global commerce is www.nanotechproject.org/inventories/consumer/.

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23

Animal feed sampling for contaminant analysis

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Abstract: In food and feed control, decisions are based on the results of analysis carried out on small samples. The results are used for assessing compliance with legal provisions, for quality control, or for technical or commercial purposes. It is pivotal to obtain a sample that is representative of an entire batch of material, from a few kilograms up to several tonnes in size. Many different approaches can be used to evaluate and minimize uncertainty in sampling. This chapter describes different sampling strategies, and deals with sampling errors and sources of uncertainty, which need to be considered in the evaluation of results for the official control of feed materials.

Key words: sampling, sampling uncertainty, sampling methods, risk assessment, official control, modelling, heterogeneity.

23.1 Introduction

23.1.1 The role of sampling

Sampling is a multifaceted process which is often implemented ineffectively and without due consideration. Correct sampling reduces the overall analytical error and is therefore directly linked to key issues such as consumer health protection and animal welfare.

The main goal of sampling is to generalize and extend the results achieved from an individual sample to the entire population with a certain level of precision. This process is generally identified as 'statistical inference'.

In food and feed control, decisions are based on the results of analysis carried out on small samples. The results are used for assessing compliance with legal provisions, for example, or for quality control, or for technical or commercial purposes. It is pivotal to obtain a sample that is representative of an entire batch of material, from a few kilograms up to several tonnes size. A proper sampling regime must be applied in order to obtain a representative sample.

Sampling is straightforward and precise only in the following three situations:

- When it is possible to analyse every single individual of a population (*census*)
- When the analyte to be controlled is *uniformly distributed* in the lot
- When the material sampled from the batch is *homogeneous*, i.e. when all units that compose the lot to be sampled are strictly identical (Gy, 2004b).

Sampling protocols are always affected by bias and lead to error. When approaching sampling strategies, it is important to identify possible sources of uncertainty, and to try to avoid the amplification of errors deriving from incorrect procedures (Gy, 1995; Petersen *et al*., 2005). Heterogeneity acts as an error generator in sampling procedures, and the resulting sampling uncertainty will add to the analytical one.

Historically, sampling uncertainty has not been taken into consideration, with only analytical uncertainty being covered in the literature (GUM, 1993; ISO, 1993, 1998; EURACHEM/CITAC, 2007). 'Sampling is a scientific holistic process approach' (Gy, 2004a) that often suffers from a lack of attention. Generally, the analyst is not responsible for the origin of the sample. However, the interpretation of the results and the overall (total) uncertainty include both the sampling error and the analytical error, where the evaluation of analytical uncertainty is a routine exercise but the evaluation of sampling uncertainty is not.

The setting up of sampling procedures in different fields is covered by regulations and guidelines worldwide. By contrast, procedures for estimating sampling uncertainty are scarce and not appropriately divulged; only the EURACHEM guidance (EURACHEM/CITAC, 2007) can be considered a sound and complete reference.

23.1.2 Presenting the four Ws (why, what, when, where) and one H (how) of sampling

An ideal, holistic sampling plan not only comprises the technical process of performing a correct sampling procedure, but also adopts cost–benefit decisions. *Why* is a sampling action needed? *What* commodity needs to be sampled? *When* is the appropriate time to perform sampling? *Where* is the best place to gather the most constructive information? The answers to these questions must be seen as a decision-making flow process, with the aim of avoiding loss of time and money, and of making the process fit-for-purpose (Miraglia *et al*., 2005).

Sampling processes can be split into the following stages:

1. The first step consists of deciding '*why*, *what, where* and *when'* to collect samples. Health and trade issues represent the overall reasons *why* samples need to be collected. *Where* and *when* to collect samples depends on the practical purpose for which they are destined. *Where* to collect

samples refers to the selection of the sites where sampling should be done (ship, dock, container, farm, stable and so on, up to the retail market). *When* to collect samples refers to the purpose of the collection, such as mandatory or targeted investigations according to national control plans.

2. The second step consists of establishing *how* samples should be collected in order to ensure they are representative of the lot under investigation. The sampling methodology should be chosen based on a 'fit-for-purpose' approach.

Obviously, providing correct sampling is performed, the quality and consequently the reliability of the data are heavily dependent on the available resources and on the skill of the people involved.

In order to define an appropriate sampling plan, knowledge of the distribution of contaminated units within the bulk is essential. Some contaminants that are heterogeneously distributed throughout the lot, such as mycotoxins, need special efforts in order to avoid incorrect sampling plans. Milling, comminuting, and homogenizing steps are generally useful for these kinds of heterogeneously distributed contaminants in order to decrease sampling error.

In conclusion, an effective sampling plan should consist of the identification of the sites and number of sites *where* samples need to be collected, and of a reliable evaluation of the status of contamination at that site or sites.

23.1.3 International norms and legislative provisions

Quoting the reference regulation for official controls, Regulation (EC) No. 882/2004 of the European Parliament and of the Council, 'feed and food should be safe and wholesome' (Commission Regulation 882/2004/EC). To achieve this purpose, official control has implemented routine surveillance checks and inspections, verifications, and audits of sampling and testing. Community legislation has a number of rules to ensure that these controls are carried out to a sufficiently high standard.

Tables 23.1 and 23.2 present the mandatory and voluntary norms on sampling, from European and international organizations, which must be put in place. For each example, specific sampling plans for different commodities are presented.

To date, harmonizing definitions and terms has been a big challenge, and despite all attempts, some differences still exist. *Lot*, *sub-lot*, *incremental sample*, *aggregate sample* and *laboratory sample* are coherent in almost all Regulations (Commission Regulations 401/2006/EC, 1882/2006/EC, 1883/2006/EC and 333/2007/EC). However, alternative terminology is still sometimes used, such as *final sample* for *laboratory sample* in Commission Regulation 152/2009/EC and *bulk sample* instead of *aggregate sample* in Commission Recommendation 787/2004/EC. This can lead to conflicting interpretations between the involved parties and a harmonized nomenclature is urgently needed.

Table 23.1 Legislative norms on sampling in the European Union

Name	Commodity	Analyte	Reference
For levels of mycotoxins in certain foodstuff	Foodstuff	Mycotoxins	401/2006
For levels of heavy metals, 3-MCPD, inorganic tin and benzo(a)pyrene levels in certain foodstuff	Foodstuff	Heavy metals	333/2007
For dioxin and PCB levels in certain foodstuff	Foodstuff	Dioxins and PCBs	1883/2006
For levels of nitrates in certain foodstuff	Foodstuff	Nitrates	1882/2006
For levels of certain contaminants in feed	Feed	Contaminants	152/2009
For sampling and detection of genetically modified organisms and material produced from genetically modified organisms	Food and feed	GMO	787/2004
Establishing Community methods of sampling for the official control of pesticide residues in and on products of plant and animal origin and repealing Directive 79/700/EEC	Food and feed	Pesticides	2002/63/EC
On microbiological criteria for foodstuffs	Foodstuffs	Micro-organisms	2073/2005

In all the legal provisions mentioned above, it is stated that sampling shall be performed by authorized personnel, appropriately trained, as designated by the Member State. Efforts are expected from Member States regarding the establishment of training courses, which should enable staff to undertake official control activities in a competent manner. Precautions must be taken during the course of sampling in order to avoid influencing the analytical results and making the aggregate samples unrepresentative. In addition, all the measures necessary to ensure the safety of the sampling operators should be taken.

The existing legislation provides suggestions on how to collect incremental samples distributed throughout the lot or sub-lot, and on how to prepare the aggregate sample. Replicate samples for enforcement, defence and reference purposes are also a matter of Regulation commitments; replicate samples shall be taken from the homogenized aggregate sample, unless such procedure conflicts with Member States' rules as regard the rights of the food business operator. The size of the laboratory samples for enforcement shall be sufficient to allow at least for duplicate analyses.

Table 23.2 Norms at international organizations

Organization	Commodity	Analyte	Reference
FAO	Peanuts and corn	Aflatoxins	FAO Food and Nutrition Paper No. 55, 1993
FAO/WHO Proposed draft sampling plan for aflatoxin contamination in almonds, brazil nuts, hazelnuts and pistachios	Almonds, brazil nuts, hazelnuts and pistachios	Aflatoxins	CX/FAC 05/37/23
FAO/WHO Codex standard for peanuts	Peanuts	Any	CODEX STAN 200-1995
FAO/WHO General guidelines on sampling	Any	Any	CAC/GL 50-2004
ISTA Handbook on Seed Sampling	Seed	Any	2nd edition, 2004
FDA/USDA	GIPSA *Grain Inspection Handbook – Book I Grain Sampling*	Any	7 July 1995
ISO	Cereals and cereal products – sampling	Any	24333/2009
NMKL (Nordic Committee on Food Analysis)	*Guide on Sampling for Analysis of Foods*		Procedure No. 12

In addition to the above-mentioned legislative provisions, the Commission has also issued a certain number of guidance documents for particularly complicated situations. Mycotoxins, for example, are the subject of two important guidance documents due to their food safety concerns (aflatoxin B1 is the most potent carcinogenic substance) and high heterogeneity distribution. These documents are a useful tool for improving the interpretation of the mandatory legislation from a pragmatic point of view, supplying technical provisions applicable to the control of aflatoxins and other mycotoxins in food products.

Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins (DG SANCO, 2010)
This document is focused mainly on the official control of aflatoxin contamination in food products subject to Commission Regulation 1152/2009/

EC. It imposes special conditions governing the import of certain foodstuffs from certain countries due to contamination risk by aflatoxins. Nevertheless, the provisions in this guidance document are also applicable, where relevant, to the control of aflatoxins in food products not subject to Commission Regulation 1152/2009/EC.

Guidance document for the sampling of cereals for mycotoxins (DG SANCO, 2009)

This document provides technical guidance for sampling lots where the sampling provisions as provided for Commission Regulation 401/2006/EC are very challenging, such as large lots of cereals stored in crucial sampling sites (silos, warehouses, ship holds, etc.).

The guidance document on official controls, under Regulation (EC) No. 882/2004, concerning microbiological sampling and testing of foodstuffs should also be mentioned here (DG SANCO, 2006).

The guidance is mainly directed at Competent Authorities carrying out official controls, and aims to give guidance on official sampling, requirements of official laboratories, analytical methods for official samples, and microbiological criteria applied to official samples in reference to Regulation 882/2004/EC.

Lastly, it should be stressed that samples must be not only analytical but also juridical. Therefore, any sample should be drawn following the so-called good sampling practices (GSP), meaning that:

- The sampling operators should handle the samples hygienically and with appropriate devices in order to avoid external contamination of the sample.
- The sampling operators should have adequate individual safety protection tools.
- The sampling devices and other required tools should be strictly controlled and spotless, and must be disinfected for each sample collection in order to avoid cross-contamination.
- Feeds should be stored in adequate containers that protect the sample from sun exposure and ensure the correct storage of the laboratory sample.
- Seals should be inviolable and report the sign of the body that performed the sampling.
- The sample packaging should ensure perfect conservation of the official sample.

23.2 Methods of sample selection

23.2.1 Terms and definitions

Lot (ISO/FDIS 24333/2009, ISO 13690/1999, ISO 6644/2002)

'Identify quantity of material from which a sample can be taken and controlled to determine one (or several) characteristic(s).'

'Stated portion of the consignment whose quality is to be asserted'.
'Stated portion of a consignment.'

Sample (ISO 3534-1/1993, ISO 11074-2/1998)

'A portion of material selected from a larger quantity of material.'

Sampling error (ISO/FDIS 24333/2009, Särndal et al., 2003)

'[Cereals] that part of the total estimation of error of a characteristic due to the heterogeneity of the characteristics, the nature of sampling and to known and acceptable deficiencies in the sampling plan.'
'Sampling error is the error caused by observing a sample instead of the whole population.'

Increment (ISO/FDIS 24333/2009, ISO 13690/1999, ISO 6644/2002)

'Amount of material taken at one time at each individual sampling point throughout a lot.'
'Small equal quantity of grain taken from each individual sampling point in the lot, throughout the full depth of the lot.'
'Small quantity taken from the product stream during a stated short period of time.'

Population or target population (Särndal et al., 2003)

'The target population is the set of elements about which information is wanted and parameter estimates are required.'

Bias (ISO 3534-1/1993)

'The difference between the expectation of the test result and an accepted reference value.'
Note: Bias is a measure of the total systematic error as contrasted to random error.

Sampling bias (ISO 3534-1/1993)

'A systematic error causing some members of the population to be less likely to be included than others. It results in a biased sample, a non-random sample of a population (or non-human factors) in which all participants are not equally balanced or objectively represented.'

Representative sample (ISO 11074-2/1998)

'Sample resulting from a sampling plan that can be expected to reflect adequately the properties of interest in the parent population.'

Uncertainty (EURACHEM/CITAC, 2007)

'Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand.'

Sampling uncertainty (EURACHEM/CITAC, 2007)

'The part of the total measurement uncertainty attributable to sampling.'

Analytical uncertainty or measurement uncertainty (JCGM, 2008)

'Non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurement, based on the information used.' Analytical uncertainty refers to the uncertainty associated with analytical results.

23.2.2 Probabilistic and non-probabilistic sampling schemes

Sampling involves the selection of a set of units from a population, and the extrapolation of knowledge about the population itself. Sampling operators collect information using a variety of methods, which are either probability-based or non-probability-based. The former involves any procedure based on the probabilistic principles of selection; the latter implies elements of judgement in the act of selection of a sample.

In a probabilistic sampling procedure, every unit has a specific probability (larger than zero) to be selected in the sample, and selection is random. A good 'random' sample is representative of the target population, and therefore reflects the characteristics of that population. The advantage of using a random sample is the absence of both systematic and sampling bias.

Probability sampling procedures include simple random sampling, systematic random sampling, stratified random sampling, cluster sampling and multistage sampling.

Non-probabilistic sampling is performed without implementing random selection methods. The probability of collection will therefore differ for different units within the population. Since probabilistic sampling is quite often not possible, non-probabilistic sampling can be performed under the rationale of specific practical criteria. A criterion of availability, for example, may help to implement the sample selection (Särndal *et al*., 2003).

The inconvenience of non-probabilistic sampling is that there is no chance to assess sampling errors, since these methods create sampling bias that increases unpredictably, rendering overall error identification almost impossible.

Simple random sampling

Every sampling technique implies the creation of a sample by the selection of n units from the target population of N units. In simple random sampling,

a subset of units is randomly chosen from the target population. Each unit has the same probability of being chosen, and so the sample is unbiased. The selection of each unit is independent.

In theory, simple random sampling is easy to implement and is the best possible condition for recording statistics. However, the target population needs to be defined and each element (frame or list of units) numbered. In practice, all the units composing the population are often not known, hence it may be expensive to conduct this method over a large population (Särndal *et al*., 2003; Foreman, 1991). Furthermore, there are specific situations in which it is impossible to implement this probabilistic method. If the lot is stored in bulk and only static sampling is possible, simple random sampling cannot be correctly applied.

Systematic random sampling

As with simple random sampling, the target population must be defined. Systematic random sampling is obtained by calculating a sampling interval (in terms of time, sampling frequency, etc.) and using this interval to select the units to be included in the sample. It is the typical method used for flowing materials (during loading or off-loading of materials, production of commodities on a stream) or when an ordered frame of the units of the populations is available.

The sampling interval k is calculated as shown in equation [23.1]:

$$k = \frac{\text{population size}}{\text{sample size}} \qquad 23.1$$

Again, the random selection guarantees that every unit has the same probability of being selected, but not all the sample has the same probability of being selected.

This technique is easy to implement and produces samples that are representative of the population. It is only if the population shows a hidden periodic trait for the characteristic under study, and the sampling interval matches the periodicity of the trait, that this sampling technique does not guarantee the representativeness of the sample.

This sampling method is much more feasible and effective in certain situations than simple random sampling. It allows probabilistic sampling of materials in bulk that could not be sampled correctly by other methods. The technique is particularly convenient for off-loading items, because it ensures that samples are collected from the beginning, middle and the end of the flow (Särndal *et al*., 2003; Foreman, 1991).

Stratified random sampling

With stratified random sampling, the population is first divided into a number of subgroups (strata), according to particular characteristics. A random selection from each stratum is then performed. The strata are defined through a specific attribute shared by all the units of the strata, i.e.

every unit in the population must be assigned to only one group or stratum, and no unit can be excluded.

This sampling technique is useful when the variable of interest has different distributions in the subgroups defined by stratification. A separate simple random sample is chosen from each stratum and all the samples are collected to form the full sample.

For example, if the aim is to study contamination by aflatoxins in cornfields in a particular region, it could be crucial to take into consideration all the varieties of corn that are cultivated there. If 95% of the crop fields are cultivated with one variety, simple random sampling might take samples only from fields with that variety. By contrast, stratified random sampling allows drawing a fixed percentage of units from every stratum (corn variety), ensuring that each stratum is included in the sample.

A stratified sample achieves greater precision than a simple random sample of the same size. Moreover, a smaller sample may be sufficient, making the method more practical and cost-effective. The main disadvantage is that the process of stratification may be difficult and costly to implement if it requires accurate information about the population. It can also be complex to organize and produce results that are difficult to analyse (Särndal *et al*., 2003; Foreman, 1991).

Cluster sampling

Cluster sampling is usually used if the target population is particularly large, and is difficult to sample with other techniques. In cluster sampling, the population is divided into groups (clusters) and each element of the population is assigned to only one cluster. Using this technique, clusters are sampled by simple random sampling or some other sampling methods.

For example, in order to obtain a sample of companies in a region, cluster sampling is implemented in two steps. Firstly a sample of cities (clusters) from the region is selected, and then all the companies of the selected cities are collected in the final sample. The technique works best when most of the variation in the population is within the groups, not between them.

The main difference between cluster sampling and stratified sampling is that while the cluster is treated as the sampling unit, and only the selected clusters are studied, in stratified sampling a random sample is drawn from each of the strata.

Because it only samples clusters of the population, this technique is cheap, quick and easy. Instead of sampling an entire population, limited resources can be allocated to the few randomly selected clusters or areas. Unfortunately, using cluster sampling can result in a high sampling error, if a relevant proportion of the population is left out of the sample (Särndal *et al*., 2003; Foreman, 1991).

Multi-stage sampling

Multi-stage sampling is a particular form of cluster sampling involving a combination of different techniques; it is used when several levels of units are inserted in one another. Samples are taken in a series of steps, and a sample of units is selected from each level. The first set of units (clusters) (e.g. nations) are randomly taken from the population available for sampling at the first stage, and the second set of units are selected only in the clusters selected at the first stage (e.g. cities) and so on. The peculiar aspect of this sampling technique is that a random sample of units is selected at every level.

Multi-stage sampling has the convenience of cost and speed, because it only needs a list of clusters and individuals in selected clusters to be performed in every level. Conversely it is less accurate than systematic random sampling of the same size (but more accurate for the same cost).

This sampling method is used for economic reasons, when is not possible to have a complete list of all the units of the target population (Särndal *et al*., 2003; Foreman, 1991).

23.2.3 Dynamic versus static methods

The implementation of sampling plans depends on the practicalities of performing the procedure. Sampling plans may be categorized into dynamic and static methods.

A wide number of variables can affect accuracy, such as the facility of the sampling site, the availability of sampling tools and apparatus, the accessibility of human and financial resources, and the time, type, shape, amount and size of items to be sampled. All these variables are crucial in deciding whether the sampling method will be dynamic or static.

As a general rule, dynamic sampling should always be preferred to static. Sampling a flowing stream allows probabilistic sampling, and so guarantees representativeness, which static sampling does not always do. Indeed, in certain circumstances probabilistic sampling can be only performed in dynamic conditions to guarantee that any incremental sample or unit has the same probability of being drawn.

Dynamic sampling can be performed on material streaming through a conveying system or in free-fall from a spout, chute, truck or ship's hold. Usually, it allows collection of incremental samples from different layers in the lot. Hence, dynamic sampling makes it possible to draw samples continuously at regular and pre-determined intervals, from the beginning to the end of the production process or the offloading of the lot. Moreover, it is safer for the operator than, for example, attempting to perform static sampling in the hold of a ship.

Dynamic sampling procedures are fully described in ISO/FDIS 24333/2009 which replaces ISO 6644/2002 related to flowing cereals and milled cereal products. ISO/FDIS 24333/2009 specifies the requirements for both dynamic and static sampling, by manual or mechanical means, for

assessment of lot quality and conditions of cereals and cereal products. It is applicable for heterogeneously distributed contaminants.

A simple equation [23.2] can be used to calculate the frequency of sample collection:

$$SF = \frac{Off\_T}{n} \qquad 23.2$$

where

SF = sampling frequency, in minutes
Off_T = offloading time, in minutes
n = total number of incremental samples to be collected.

As for static lots, typical examples are products in containers, silos, trucks, warehouses, and stored in sacks or at retail.

As previously described, static lots often entail circumstances that make it impossible to guarantee the same probability for every item to be sampled. Probabilistic sampling is therefore not always possible. Typical examples include circumstances where the probe length is shorter than the depth of the truck, van or ship hold, or when units (e.g. commodities stored in warehouses, grains in big silos or in large heaps) are unreachable. In these cases the minimum length of a probe should be 2 metres, in order to improve accessibility and guarantee representative sampling as far as possible.

Sampling in static conditions is much more challenging than in dynamic, since it involves safety hazards (unless using automatic probes) and practical difficulties, is time consuming, and requires trained personnel. Thus static sampling is preferable only when all the units of the lot are physically reachable, or when dynamic sampling is unfeasible.

Sampling of silos is unwise in static conditions, due to the impossibility of collecting incremental samples from a static mass in a representative way. Therefore, a silo must be sampled only during uploading or offloading.

In the case of sacks or bags stored in a warehouse, sampling is usually performed by randomly selecting a number of sacks or bags from which incremental samples are collected by probes. This procedure, although feasible, is rather defective in that the choice of the sacks is not random and the sample is drawn only from the reachable side of the sack or bag. If possible, this procedure should be corrected by (1) numbering all the sacks or bags forming the lot, (2) selecting a requested number of sacks or bags at random, and (3) collecting the incremental samples from different points of the sack or bag.

Static sampling procedures are fully described in ISO/FDIS 24333/2009 which replaces ISO 13690/1999 relating to sampling of static batches of cereals, pulses and milled products. ISO/FDIS 24333/2009 specifically describes the minimum mass and the methodology to be used for different kinds of contaminants in cereals and cereal products. Examples of suitable instruments for sampling static products and dividing samples are also reported.

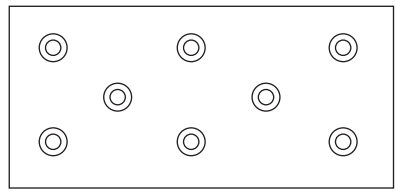

Fig. 23.1 Examples of distribution of sampling points following ISO 24333:2009 for trucks or railcars from 15 to 30 metric tons. Eight sampling points.

More often, sampling sites in trucks or railroad cars are strictly dependent on specific agreements between the parties, and the number and size of incremental samples depend on the lot size, according to the norm ISO/FDIS 24333/2009. For instance, for 15–30 tonne trailer-trucks, a minimum of eight incremental samples in eight different sampling points (Fig. 23.1) should be considered.

For static sampling procedures, different probes are used: probes with various openings, probes with open or closed shafts with one or several openings, probes with several sequentially staggered openings, and probes with concentric, cut-type, gravity-type openings with extension rods and T-shaped handles. Specific instruments are also used for sacks or bags, such as tapered, walking stick, and cone-shaped sampling probes.

When sampling using probes, the relationship between the openings and the number of incremental samples to be collected should be regulated. As a general principle, a single opening can be considered as one incremental sample, provided that the minimum amount of material is collected in each opening (100 grams for cereals and 200 grams for oilseeds). However, following DG SANCO indications, a probe should have a maximum distance of 0.5 metres between the openings (DG SANCO, 2010).

Any of the above-mentioned sampling tools can be used both for cereals in grains and for milled derived products.

23.2.4 Manual versus automatic methods

The main advantages of using the automatic approach lie in the fact that it reduces costs in terms of personnel involvement, and allows full control of the whole sampling procedure together with a rationalization of safety precautions. Moreover, whenever possible, the use of GPS instrumentation facilitates monitoring of the process along the entire sampling flux of operations. Generally, because the process is automated, precision is also improved.

Many types of equipment are commercially available for the sampling of moving streams of material. These include in-line samplers like the *Pelican* and the *Ellis Cup,* which cross the stream, allowing extraction of incremental samples. The *diverter-type* is another example, and is probably the best device for obtaining representative samples from bulk grain. It is

designed to take a complete cross-section by cutting through the stream of grain. Extracted grain may be fed directly into a secondary sampler, which reduces the sample to a manageable size. The s*ample dividers* should also be mentioned, since they are used to reduced the sample in size and distribute it into a specific adapter.

Other automatic apparatus includes the cross-cut samplers, such as exporter, designed for high volumes; rotary, designed for vertical gravity flow sprouts and chutes; pneumatic line samplers, suitable for flour, pellet, granules and heavy viscosity liquids; and belt end samplers with pelican-type diverters. All of these can be regulated for collecting samples continuously and at preset intervals.

From the technical side, as a general rule, diverter caps should have an opening two or three times larger than the dimensions of the items in the lot (Council of Agricultural Science and Technology, CAST, 2003).

When implementing manual methods, a complete set of tools should be available on the site, especially during inspections or official control activities, which must be carried out in the presence of an official inspector of the Competent Authority equipped with all necessary devices. Common tools used in manual sampling are *hand-scoops*, *sampling spears*, *sleeve samplers* (for sampling large volumes at great depths), and *sack-bag samplers* (for taking samples through the side of a sack or bag). In addition, it is highly recommended that operators are supplied with personal protective equipment (PPE), such as worker overalls, gloves, goggles, safety helmets, masks and boots in order to maximize safety.

Manual sampling still remains the most common procedure, notwithstanding its significant requirements in terms of human and financial resources, safety measures, training of personnel and time, and its higher contribution to the uncertainty budget.

23.3 Designing sampling plans

A sampling plan must take into account some crucial points, such as the distribution of the variable under study in the lot, the lot size, the incremental sample size, and the number of aggregate samples to be drawn.

23.3.1 Heterogeneous versus homogeneous distribution of analyses in a lot

IUPAC defines both homogeneity and heterogeneity as 'the degree to which a property or constituent is uniformly distributed throughout a quantity of material' (IUPAC, 1990; ISO 11074-2/1998). Many sources are relevant in defining sampling uncertainty, but heterogeneity is commonly considered the most significant, indeed 'the degree of heterogeneity is the determining factor of sampling error', as asserted by IUPAC (IUPAC, 1990).

A certain grade of heterogeneity always exists in all kinds of material. Heterogeneity can be divided in two parts: (1) constitutive heterogeneity (CH), which takes into account the physical and chemical differences in the material; and (2) distributional heterogeneity (DH), which refers to the spatial distribution of the material due to possible stratification and segregation within the batch (Petersen and Esbensen, 2005).

Many sampling plans are based on assumptions about the distribution of the variable under study, and random distribution is frequently assumed. These assumptions cannot be granted in every case, especially if the relevant variable is heterogeneously distributed, e.g. in lots contaminated with mycotoxins, or containing GMOs. If the real distribution is far from being random this should be taken into account, since the wrong assumption can jeopardize results.

The geostatistics theory can be used to overcome problems related to the distribution of variables. Geostatistic models highlight spatial patterns where it is reasonable to think that the random distribution is not realistic (Paoletti *et al*., 2006). Specifically, geostatistics takes into account the heterogeneity of a lot and the autocorrelation among the units, and explores situations where a non-random distribution exists.

Spatial correlation or autocorrelation appears if units at proximal locations are correlated, hence close units are more related than distant ones. It may also be described as the variation observed between units as a function of the distance in time or space between them (Petersen and Esbensen, 2005).

The study of autocorrelation and heterogeneity can be crucial when designing a sample plan, i.e. examining the process variation over time or space. For example, the pattern of variability in the process or variable studied, the number of incremental samples, and the frequency of sampling, are gathered from autocorrelation studies and their derived functions (as variograms) (Petersen and Esbensen, 2005). A variogram is a function that describes the spatial dependence of units in relation to space or time, and may help in optimizing sampling schemes in order to reduce the total sampling error.

Furthermore, resampling techniques attempt to face the problem of heterogeneity (see below for a description) (Good, 2006). These techniques are iterative algorithms that randomly extract samples from the available data. The main advantage of using these methods is that they are not parametrical, and do not require assumptions concerning the distribution of the variable studied. The technique can therefore be applied both when heterogeneity occurs and when no information on distribution is available.

23.3.2 Number and size of incremental samples; weight of aggregate sample

The major topics concerning the design of a sampling plan are representativeness, minimization of sampling bias, and the determination of sampling

size. All studies have economic and practical constraints and, in the case of designing a sampling plan, the need to balance statistical, feasibility and economic criteria is imperative.

As a general rule, increasing the sampling size has a positive influence on the study (Särndal *et al*., 2003; Foreman, 1991). In order to decide how large a sample should be, it is important to define the target population, the frame (i.e. the list of all the units of the target population), and the dimension of the lot. Sample size typically depends on the nature of the study or analysis, the desired precision of the estimates, the kind and number of comparisons that will be made, the number of variables that have to be examined simultaneously, and the heterogeneity of the variables.

In determining sample size, the goal is to include sufficient numbers of units to guarantee statistical significance of results or accurate estimations. Using a scarce number of units may result in wasted time, useless effort, and lost money, due to the complete lack of representativeness. On the other hand, using too many units may result in costly answers that could have been produced with fewer subjects, less time and less money.

With continuous variables (e.g. contamination level, concentration level, depth and weight values) or fractions (e.g. number of broken items in a lot), it is possible to determine the sample size by means of specific formulas, once the desired precision and level of significance have been established and an estimate of the variability of the population has been assessed. Assuming that the distribution of the sample means is normal, the sample size (n) is computed, for a certain precision, according to the following formula:

$$n = \frac{v_1}{\Delta^2} * Z^2 \qquad 23.3$$

where

v_1 = variance among individual units
Δ = deviation of sample mean about the population mean
Z = critical value of the normal distribution at 95% level of confidence.

The European Commission has provided several regulations for official control of specific analytes (as mycotoxins, GMO, etc.), giving indications about sampling protocols, number and weight of incremental samples, etc. The main criterion was to correlate the size of lots or sub-lots with the number of incremental and aggregate samples (ISO/FDIS 24333/2009; Commission Regulation 401/2006/EC; Commission Regulation 152/2009/EC; Commission Recommendation 787/2004/EC; Commission Directive 2002/63/EC).

So far as feed legislation is concerned, the official reference for sampling is Regulation (EC) No. 152/2009 of 27 January 2009, which lays down the methods of sampling and analysis for the official control of feed. This Regulation replaced all 35 implementing acts to Directive 70/373/EEC, now replaced by EC Regulation 882/2004, by a single Regulation with the aim

of improving coordination. This provision concerns sampling rules for the determination of constituents, additives and undesirable substances, with the exception of residues of microorganisms (Regulation (EC) 2073/2005) and pesticides (Commission Directive 2002/63/EC).

A description of sampling protocol is given in Annex I of the Regulation, where the following definitions were set:

- *Sampled portion*: A quantity of product constituting a unit, and having characteristics presumed to be uniform.
- *Incremental sample*: A quantity taken from one point in the sampled portion.
- *Aggregate sample*: An aggregate of incremental samples taken from the same sampled portion.
- *Reduced sample*: A representative part of the aggregate sample, obtained from the latter by a process of reduction.
- *Final sample*: A part of the reduced sample or of the homogenized aggregate sample.

From a pragmatic point of view, no substantial differences were introduced with respect to the previous Commission Directive 76/371/EEC.

The Regulation describes different procedures for analytes homogeneously and non-homogeneously distributed in the lot. In both cases, the protocol takes the following parameters into consideration: lot size, number and weight of incremental samples, number of aggregate samples, and number of final samples. It is important to note that a strict correlation among all the mentioned parameters was considered, with more stringent rules for analytes non-homogeneously distributed throughout the lot, as in the case of aflatoxins, rye ergot and crotalaria.

In the Regulation, manual and/or mechanical sampling is mentioned and considered fit for purpose.

For homogeneously distributed analytes, incremental samples must be taken at random throughout the whole sampled portion, and they must be of approximately equal sizes. Where appropriate, sampling may be carried out when the sampled portion is being moved (loading or unloading).

Indications of sampling for loose, packaged, semi-liquid and liquid feeds, block feeds and mineral licks are reported.

The overall sequence, according to the rules of Regulation 152/2009, is as follows:

1. Assessment of the nature of the analyte (homogeneously or non-homogeneously distributed in the lot)
2. Evaluation of the lot or sub-lot size
3. Evaluation of the number and size of incremental samples
4. Gathering of the incremental samples to form one or more than one aggregate sample

5. Formation of the reduced sample after the homogenization of the aggregate sample
6. Formation of the final sample from the homogenized reduced sample.

For food products, the official references reflect the same criteria as described for feeds.

More specifically, the following Regulations are currently in place for different analytes:

- Mycotoxins – Regulation (EC) 178/2010 amending Regulation 401/2006
- Lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene – Regulation (EC) 333/2007
- Nitrates – Regulation (EC) 1882/2006
- Dioxins and dioxin-like PCBs – Regulation (EC) 1883/2006.

Considering mycotoxins as the most crucial topic in terms of sampling, the description of what was set out in Regulations 178/2010 and 401/2006 will be reported. In the case of cereals and cereal products the main indications of the Regulation may be summarized as follows. To sample lots traded in individual packs, such as sacks, bags and retail packing, the following formula may be used:

$$SF = \frac{WL * WIS}{WAS * WIP} \qquad 23.4$$

where

SF = sampling frequency
WL = weight of the lot, in kg
WIS = weight of the incremental sample, in kg
WAS = weight of the aggregate sample, in kg
WIP = weight of the individual packing, in kg.

The sampling frequency (n) indicates that for every n packs one incremental sample is drawn. The weight of the incremental sample must be 100 grams, unless otherwise indicated by Regulation 401/2006 for specific commodities such as dried figs (300 g) and groundnuts (200 g).

According to Regulation (EC) 401/2006 for lots of cereals and cereal products ≥50 tonnes, if the sub-lot cannot be separated physically, each lot must be subdivided into sub-lots following the outline reported in Table 23.3 and sampled separately. A minimum of 100 incremental samples should be taken from the lot, providing an aggregate sample of 10 kg. For lots of cereals and cereal products <50 tonnes, the number of incremental samples varies between 10 and 100 (aggregate sample between 1 and 10 kg) depending on the lot weight (Table 23.4).

If the single retail pack is widely more than 100 g, for example for packs of 500 g or 1 kg, the global sample may be obtained from a reduced number of incremental samples on condition that the weight of the global sample

Table 23.3 General survey of the method of sampling for cereals and cereal products

Commodity	Lot weight (tonnes)	Weight or number of sublots	Number of incremental samples	Aggregate sample weight (kg)
Cereals and cereal products	≥1500	500 tonnes	100	10
	>300 and <1500	3 sublots	100	10
	≥50 and ≤300	100 tonnes	100	10
	<50	–	3–100*	1–10

* Depending on the lot weight (Table 23.4).

Table 23.4 Number of incremental samples to be taken depending on the weight of the lot of cereals and cereal products

Lot weight (tonnes)	Number of incremental samples	Aggregate sample weight (kg)
≤0.05	3	1
>0.05 and ≤0.5	5	1
>0.5 and ≤1	10	1
>1 and ≤3	20	2
>3 and ≤10	40	4
>10 and ≤20	60	6
>20 and ≤50	100	10

is compliant with Regulation (EC) 401/2006. This Regulation also provides rules for other food products such as dried fruit, including dried vine fruit and derived products, with the exception of dried figs, wine, milk, spices and coffee.

Subsidiary technical guidelines for correct implementation of Regulation (EC) 401/2006 have been provided by the European Commission (DG SANCO, 2009). The guidance refers to large lots of cereals stored in various locations where sampling provisions are hard to apply. The following sampling sites are considered: lots/batches in lorries, trucks, train wagons (not exceeding a size of 500 tonnes), ships' holds (dynamic, static sampling), warehouses (dynamic, static sampling), silos (dynamic, static sampling), and bulk consignments in closed containers. The main situations covered by the guidelines are summarized in Table 23.5.

Furthermore, in the annex of the guidelines, a number of sampling devices used on flowing grain (such as crosscut sampling devices, full-flow diverter-type sampling devices, rotating cup sampling devices and bucket elevator sampling devices) are covered. Examples of instruments used to sample static products and divide samples (such as manual concentric

Table 23.5 Reference provisions for calculating the number of incremental samples

Storage container and number of incremental samples (IS)	Sampling equipment	Notes
Silos For lots > 500 tonnes:number of IS = 100 + √tonnes, e.g. for a consignment of 10,000 t: IS = 100 + √10,000 = 200 *Example 1*: Sampling probe of 2 metres with four apertures, collected quantity 100–150 g per aperture = 4 incremental samples	The proper kind of sampling device should be chosen taking into account the product to be sampled, the quantity required and the containers. Probes with several apertures may be used. Every aperture is considered as one incremental sample, min. 100 g for cereals and 200 g for oilseeds. A maximum of one incremental sample per 0.5 m length of sampling probe can be accepted.	Vertical silos can store 500–20,000 tonnes per cell with 4–50 cells, and can be 10–50 metres high.
Silos easily accessible from above	Sampling must be representative of the accessible part of the consignment for the result to be considered valid for the whole batch in the silo. The sampling probe must be at least 2 metres long.	In the case of non-compliance and if the representativeness of such sampling is questioned, the operator can request at his cost an official representative sampling of the whole consignment in the silo involving a movement of the whole lot from one silo to another.
Silos not accessible from above (closed cylindrical silos), size < 100 tonnes	Sampling of such batches should be done in flow (i.d. moving to an empty silo) if it is not possible to take incremental samples across the whole batch.	In the case of non-compliance and if the representativeness of such sampling is questioned, the operator can request at his cost an official representative sampling of the whole consignment in the silo involving a movement of the whole lot from one silo to another.
Silos not accessible from above (closed cylindrical silos), size > 100 tonnes	If static sampling cannot be done and the consignment cannot be moved, the operator must tell the inspector when the silo will be unloaded for sampling when the lot is in flow.	
Bulk consignments in closed containers	Can be sampled only when unloaded. Sampling may not be possible at point of import, so must be done during unloading.	

Bulk shipments and large batches in storage		Bulk shipments are commonly transported in vessels from 500 to >90,000 tonnes (Panamax, Handy-max, Handy, coasters, river barges).
Sampling of batches transported by ship by dynamic sampling *Example 2*: Batch of 10,000 t with unloading speed 500 t/h (20 hours): (a) For sampling the whole batch: IS = 100 + √10,000 = 200 (100 g each, 20 kg bulk sample) (b) For a sampled part (min. 10%) of the whole batch: IS = 100 + √1000 = 132 (150 g each, 20 kg bulk sample)	Sampling must be done per hold (an entity that can be physically separated). The sample can be taken automatically, but the presence of an inspector is necessary.	Sampling may be undertaken of part of the portion to be sampled, and is considered representative provided that at least 10% of the portion is sampled. If the operator questions the representativeness of the sampling, the whole batch will be sampled at the operator's cost.
Sampling of batches transported by ship by static sampling	Sampling must be representative of the accessible part of the consignment for the result to be considered valid for the whole batch in the ship/hold. The sampling probe must be at least 2 metres long.	In the case of non-compliance and if the representativeness of such sampling is questioned, the operator can request at his cost an official representative sampling of the whole consignment in the ship involving a movement of the whole lot from the ship into a storage facility.
Warehouses *Example 3*: For a warehouse 50 m × 30 m × 4 m, volume = 6000 m^3, capacity = *c*.4500 t, IS = 100 + √4500 = 167 incremental samples of 100 g each	Sampling must be representative of the accessible part of the consignment for the result to be considered valid for the whole batch in the warehouse. The sampling probe must be at least 2 metres long.	In the case of non-compliance and if the representativeness of such sampling is questioned, the operator can request at his cost an official representative sampling of the whole consignment in the warehouse involving a movement of the whole static lot.

Source: adapted from DG SANCO, 2009.

tapered sampling probes, mechanical sampling devices, and instruments for taking samples from sacks or bags, including bulk sacks) are also described.

23.4 Estimation of sampling uncertainty

23.4.1 Overview of approaches for estimating sampling uncertainty

Uncertainty is widely defined as a 'parameter, associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand' (EURACHEM/CITAC, 2000). It arises from a variety of sources, with sampling being one of the most significant.

According to EURACHEM uncertainty guidance (EURACHEM/CITAC, 2007), two general approaches for uncertainty estimation have been identified: the empirical approach and the theoretical one. The empirical approach (also described as the 'top-down approach') uses replication measurements to estimate uncertainty for the final result. The second approach is the modelling, theoretical, or predictive approach (also indicated as the 'bottom-up approach'), which identifies all the sources of uncertainty and then uses a model to combine and quantify them.

The theoretical approach is more generally used to estimate analytical uncertainty, but has recently begun to be used for assessing sampling uncertainty too. Estimation of the sources is obtained by empirical or other methods. The empirical approach has wider applicability and is more extensively used. For example, Whitaker and colleagues used it to evaluate sampling uncertainty in a number of different situations for diverse mycotoxins and matrices (Whitaker *et al*., 1998, 2000, 2006). It should be highlighted that both approaches can be used together to evaluate the same measurement system, and that there is no single reference for estimating sampling uncertainty.

The EURACHEM Uncertainty Guide identifies eight main categories of sources of uncertainty, two of which are sampling and sample preparation (EURACHEM/CITAC, 2000). When it comes to sampling, the sources of uncertainty originate from effects of heterogeneity; effects of specific sampling strategies; effects of movement of bulk medium; physical state of bulk, temperature and pressure effects; and effects of the sampling process on sample composition, transportation and preservation. In order to build up a mathematical model in a bottom-up approach, every source of uncertainty has to be quantified.

Top-down approaches are relatively easy and fast, and do not require previous knowledge of the materials to be sampled, but they do not take bias into account. These approaches may be seen as a rapid and economical way of achieving an approximate estimation of sampling uncertainty, or at least its major component, though it may be underestimated. A bottom-up approach is more precise in identifying all the sources of uncertainty but requires higher investments and more knowledge.

23.4.2 Theory of sampling

A good theoretical approach for identifying components of sampling uncertainty, and consequently their reduction, is given by the theory of sampling (TOS) (Gy, 1995, 2004a, b; Petersen and Esbensen, 2005; Petersen *et al.*, 2005). The TOS was conceived by Pierre Gy in the 1950s for elucidating sampling problems of particulate matrixes in the mineralogical field. The theory has proved valid in other fields too, whenever the fundamental sampling principles of TOS are obeyed.

TOS provides a methodology for the qualitative identification of sampling errors, and gives indications for their minimization and/or elimination. Once the geometry or the dimensionality (between zero and three) of the lot has been defined, it quantifies every single error contribution affecting the sampling process.

From TOS theory we understand that *correct sampling* is both *accurate* and *reproducible* and thus *representative* (Petersen *et al.*, 2005; Esbensen *et al.*, 2007). *Correct sampling* starts from the assumption that all the constituents have an equal probability of being selected, respecting the integrity of the samples (Gy, 2004a).

In TOS theory, the *geometry* of a lot must be clarified; the *dimensionality* of a lot (between zero and three) can be ascertained depending on how the sample is taken from the lot itself:

- *Zero-dimensional* (0-D) is when (1) the whole lot is taken as a sample, the sampling error being zero; or (2) the expectance value of a sample is independent of the location of the lot from which it is taken, and is completely free of spatial, physical or chronological autocorrelation between the fragments in the lot. These conditions are found where the lot is well mixed and the particles are homogeneously distributed (Petersen *et al.*, 2005).
- *One-dimensional* (1-D) is a lot that has two dimensions, leaving out the third and spatial or temporal correlation; this is the case of a flowing/moving material stream where the sample is a slice of the flowing material (Petersen *et al.*, 2005).

TOS only applies to these two dimensionalities (0-D and 1-D); 2-D (with one negligible dimension) and 3-D (when the extracted samples cannot cover any of the three dimensions) are not covered. Sometimes these can be transformed into 1-D lots, by placing them on a conveyor belt, for example (Petersen *et al.*, 2005).

For this theory, the global estimation error (GEE) is the sum of the analytical error (total analytical error, TAE) and the sampling error (total sampling error, TSE) (Petersen *et al.*, 2005):

$$\text{GEE} = \text{TSE} + \text{TAE}$$

The TSE springs from the sum of a minimum unavoidable sampling error (i.e. the correct sampling error, CSE) and a series of other contributions

that can be controlled and minimized when identified (i.e. the incorrect sampling errors, ISE):

$$TSE = CSE + ISE$$

The main problem with incorrect sampling errors is the generation of a sampling bias that increases total variance; hence ISE should be minimized from the estimation of uncertainty or possibly be eliminated (Petersen *et al*., 2005).

CSE is the sum of the fundamental sampling error (FSE) and the grouping and segregating error (GSE) (Petersen *et al*., 2005):

$$CSE = FSE + GSE$$

The FSE and GSE originate from the heterogeneity of the material: they cannot be entirely eliminated even under the assumption of a correct sampling procedure, but they can be minimized.

The FSE depends on the critical particle size within the sample. It arises from the compositional differences between particles, i.e. from the constitutional heterogeneity, and is the reason why a sampling procedure never produces two incremental samples with an entirely identical composition. For liquids, FSE is small, but for solids and powders it can be considerably larger. Nevertheless, FSE can be minimized by comminuting or crushing the material, i.e. by reducing the compositional size differences between the particles.

The GSE originates from the tendency of particles to segregate, grouping together in a lot, and can be reduced by thoroughly mixing the lot to achieve complete homogeneity.

The incorrect sampling error, ISE, results from an incorrect procedure of sampling. It can be totally avoided if sampling is carried out correctly, strictly following the sampling theory. ISE is the sum of contributions arising from the technical side of collecting samples:

$$ISE = IDE + IEE + IPE$$

The incorrect delimitation error (IDE) contribution is generated when the probability of selection is not uniform. IDE can be totally avoided if the flow stream of material has a proper velocity. The incorrect extraction error (IEE) concerns the suitable technical parameters for the proper collection of sampling increments. The suitable technical parameters for a 1-D lot have been experimentally proved (Petersen *et al*., 2004). Finally, the incorrect processing error (IPE) summarizes the contributions of cross-contamination problems during the sampling process: loss of material when producing samples: alteration in chemical composition due to incorrect treatment of the sample (loss of water, for example); alteration in physical composition (loss of fine fragments, or exposure to water or sun); involuntary faults committed by the operator; and deliberate faults aiming to compromise the representativeness of the sample.

In conclusion, the total sampling error is given by the sum of the above-mentioned contributing errors (Petersen *et al*., 2005):

$$TSE = FSE + GSE + IDE + IEE + IPE$$

23.4.3 Other techniques to estimate sampling uncertainty: resampling methods

Resampling methods are techniques used to estimate statistical parameters such as mean, median and variance by using subsets of available data or drawing random samples. They are also used to validate models and to estimate uncertainty by using random samples of the available data. The most common resampling techniques are bootstrapping, jackknifing and Monte Carlo methods (Good, 2006).

Bootstrapping is a statistical method for estimating the sampling distribution of an estimator, by sampling replacements from the original sample. It is a not an especially useful parametric technique unless it is possible to verify the assumptions needed by the parametric inference methods (Efron and Tibshirani, 1993). The jackknifing technique is performed by systematically recomputing the statistic estimate, leaving out one observation at a time from the sample set (Efron and Tibshirani, 1993). Both methods estimate the variability of a statistic computed among subsamples, rather than estimating it from parametric assumptions. The jackknife and bootstrap may yield similar results (Shao and Tu, 1996). Finally, Monte Carlo methods are algorithms based on repetition of random sampling of random or pseudo-random numbers (Good, 2006).

Recently, a novel approach to estimate the optimal sample size in experimental data collection using a resampling technique has been proposed by Confalonieri (Confalonieri *et al*., 2007). This method is based on a visual evaluation system of sample size determination, derived from a resampling based procedure (jackknife). It systematically takes subsamples of the original data set, and computes mean and standard deviation for each subsample. Using a resampling technique, this approach overcomes the typical assumptions of conventional methods. This novel approach was used to implement a program called SISSI (Sample Size Determination Routines) for the estimation of the optimal sample size in experimental data collection. SISSI is ideally created for the collection of plant and soil samples from field-grown crops, but also has more general applications.

23.5 Future trends

In order to improve the quality of sampling procedures, future activities should be focused on the following issues.

Table 23.6 Software for estimating measurement uncertainty

Name	Link	Description
ROBAN	http://www.rsc.org/Membership/Networking/InterestGroups/Analytical/AMC/Software/ROBAN.asp	Powerful modelling tool for estimating total measurement uncertainty through classic analysis of variance (ANOVA) and robust analysis of variance (RANOVA)

23.5.1 Development of statistics programs/software to evaluate sampling techniques

Various software packages are available for determining uncertainty and sample size, for studying possible spatial correlations, and for resampling techniques. Tables 23.6–23.11 illustrate some of the most widely used sampling software; each table refers to a specific field of application.

There are two possible approaches for implement resampling methods: by programming in a computer language such as C++, *R*, Resampling Stats, or SAS, or by using menu-driven programs such as Excel, SISSI, *S*-Plus, Stata or StatXact.

23.5.2 Drawbacks of implementing sampling plans

In order to consolidate rules for planning sampling procedures, including key requirements such as reliability, fast response, affordable costs and real feasibility, some issues must first be resolved.

In fact, there is no single study aimed at calculating the overall costs of sampling procedures, as set out by European and international legislation. Analysis of sampling costs should be performed, investigating the food and feed chain sector by sector, for both chemical contaminants and microbiological agents. It would then be possible to correlate sampling costs with the degree of accuracy and practical feasibility.

Another aspect to consider is the fact that, since official control requires the implementation of extremely complex and even unfeasible sampling plans, subsidiary procedures substituting the official ones are sometimes required. Moreover, sampling plans are sometimes used for different purposes from which they were originally conceived, without any evidence of their feasibility. To overcome this drawback, an effort ought to be made to create sampling procedures suitable for as broad a spectrum of analytes as possible.

Even when automatic sampling is applied, training courses and operator skill remain important issues, since these requirements can only be assured in big private companies and advanced control systems in some industrialized countries.

Table 23.7 Software to calculate the sample size/power effect

Name	Link	Description
POWER and PRECISION	http://www.power-analysis.com/home.htm	Calculates the sample size required for any level of power.
Wessa	http://www.wessa.net/stat.wasp	The Free Statistics Calculator offers sample-size calculations, descriptive and explorative analysis.
PC-Size	ftp://ftp.simtel.net/pub/simtelnet/msdos/statstcs/size102.zip	Uses exact and approximate methods for sample size/power calculations for *t*-tests, 1-way and 2-way ANOVA, simple regression, correlation, and comparison of proportions.
DSTPLAN	http://biostatistics.mdanderson.org/SoftwareDownload/SingleSoftware.aspx?Software_Id=41	Calculates sample size/power for *t*-tests, correlation, a difference in proportions, $2 \times N$ contingency tables, and various survival analysis designs.
Splus	http://www.insightful.com/products/splus/default.asp	Provides functions for sample size/power for *t*-tests and comparing proportions.
Stata	http://www.stata.com/	Has some simple built-in power and sample size functions.

Lastly, there is still a general lack of awareness of the consequences of bad sampling procedures in terms of economic losses and unsatisfactory animal and consumer health protection.

23.5.3 Alternative methods of overcoming inherent difficulties in sampling procedures

A research group recently attempted a parallel, non-invasive approach aimed at verifying whether conventional sampling procedures can be compared to alternative ones. The acoustic and optical approach was among the most promising, giving satisfactory results in terms of costs and feasibility.

Researchers at the Kaunas University of Technology (Lithuania) recently adopted the acoustic waves technique to determine deoxynivalenol directly on mixtures of unaffected and affected grains and aflatoxin-inoculated corn kernels (Juodeikiene *et al*., 2004, 2005). The basic principle of this testing is that sound, propagating through a porous structure, is attenuated, and its

Table 23.8 Software for resampling techniques

Name	Link	Description
C++	http://www.cplusplus.com/doc/tutorial/	One of the most popular programming languages ever created. It is considered as a *middle-level* language. C++ is widely used in the software industry.
R	http://cran.r-project.org/doc/manuals/R-intro.html	One of the most used programming languages for statistical computing, widely used for statistical software development and data analysis.
Resampling Stats	http://www.resample.com/content/about.shtml	Implements resampling methods in statistics (including simulations, as well as bootstrap and permutation procedures); available as an independent product, as well as for Excel and Matlab.
SAS	http://www.sas.com/	The leader software in business analytics software and services. It includes macros for resampling methods.
SISSI	http://hdl.handle.net/2434/26500	Resampling-based software for sample size determination.
S-Plus	http://stat.ethz.ch/~www/SandR.html	A high-level language and an environment for statistical analysis and graphics development, including application for resampling methods.
StatXact	http://www.cytel.com/Software/StatXact.aspx	A package for exact non-parametric statistical inference on continuous or categorical data, data analysis, modelling, visualization, data mining and fitting.

speed depends on pore size and the porosity coefficient. In this context, any changes in velocity and/or amplitude of the wave can be monitored by a spectrometer, and used to identify the presence of wholesome and shrivelled grains. In the cited study, two different pieces of equipment were used: an acoustic spectrometer with transmission, and an acoustic impulse spectrometer with transmission and reflection.

This method gave satisfactory results, showing a linear relationship between scabby wheat grains and the amplitude of the acoustic signal. In

Table 23.9 Software to study sampling strategies and distribution properties

Name	Link	Description
KeSTE	http://www.sipeaa.it/tools/KeSTE/What_use_for_KeSTE.htm	An exploratory tool developed to investigate the possible consequences of different sampling strategies on the structure and properties of bulk samples when contaminants are not randomly distributed. KeSTE allows the evaluation of sampling strategies on both simulated lots and on real lots, through the analyses of data collected from a maximum of 100 increments.

Table 23.10 Software for acceptance sampling plans

Name	Link	Description
OPACSA	http://www.coextra.eu/researchlive/reportage851.html	An optimization software that helps to find the cheapest and most reliable mode of analysis by subsampling to decide on the acceptance or rejection of a specific lot.
Acceptance Sampling Plan Designer	http://www.ct-yankee.com/sampplan/	A freeware program to design single-sample acceptance sampling plans.
Display TP414 Version 3	http://www.samplingplans.com/programtp414.htm	A program to design acceptance sampling plans by variables.
Display TP105 Version 3	http://www.samplingplans.com/usermanuals/tp105manual03.pdf	A program to design acceptance sampling plans by attributes.

Table 23.11 Software for spatial correlation

Name	Link	Description
SADA	http://www.tiem.utk.edu/~sada/geospatial_analysis.shtml	Package including methods for measuring spatial correlation among data.
VARIOWIN	http://www-sst.unil.ch/research/variowin/#DOWNLOAD	Software for analysis of spatial data in 2D.
GeoEAS	http://www.epa.gov/ada/csmos/models/geoeas.html	The most famous geostatistical package, implementing all the steps required in geostatistics (primary statistics, spatial correlation analysis, variogram modelling, interpolating with kringing and cross-validation).

addition, the results were in line with conventional detection methods, showing performance criteria largely within the EU provisions.

Spanjer *et al*. experimented with another automated, non-destructive method for avoiding complex and unreliable sampling procedures for bulk commodities (Spanjer *et al*., 2001). The method was based on physical and chemical excitation. A remote rapid release of contaminant residues was obtained, and vapours and particulates were determined by an inspection tunnel enclosure, which was characterized by a series of physical processes such as transient thermodynamic activation, pressure cycling and sonic shock waves. This methodology, routinely used for detecting explosives, was tested for aflatoxin detection in bulk lots of pistachio nuts. It performed well, meeting export/import quality requirements set by the European Commission for mycotoxins.

23.6 References and further reading

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24

Ensuring the safe supply of animal-derived ingredients for animal feed

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Abstract: Animal by-products are potentially important sources of nutrients for animals if they are safely sourced and processed into ingredients for animal feeds. The current status of the industry is contrasted with the historical situation, and the new initiatives taken in recent years to ensure safe supply of animal-based feeds are discussed in detail.

Key words: animal by-products, fish by-products, processed animal proteins (PAP), rendered fats, fish oil, rendering, processing, risk analysis, HACCP.

24.1 Introduction

Livestock and fishery industry by-products have been utilised in animal feeds for over a century. After the by-product raw materials are transformed into products, these may be used as ingredients by the animal feeds industry. The rendering industry focuses on the processing of by-products from terrestrial (livestock) animals and produces protein meals termed meat and bone meal (MBM) of processed animal proteins (PAP), and rendered animal fats, normally called tallow. The fish by-products industry deals with fishery by-products and fish not intended or used for human consumption and produces protein meals termed fishmeals and fats called fish oils. This chapter will consider the current requirements for ensuring the safe supply of these animal-derived ingredients for animal-based feeds.

In reaching conclusions about the current levels of safety, it is important to consider the scope of the industry and its history through the twentieth century. The most significant changes in these sectors have resulted from the bovine spongiform encephalopathy (BSE) epidemics, first in the UK

and later in the rest of Europe. The way in which legislation and the rendering industry have interacted from 1996 in the UK and from 2001 in Europe will be discussed in some detail. The role of risk assessment will be considered in relation to the categorisation of animal by-products and their subsequent processes, and lastly their uses, including in animal feeds, will be discussed. Global standards, which have slowly become more harmonised, are presented and discussed. In terms of regulation and controls, however, Europe still leads the way. Therefore, a summary of the current EU regulations will be given together with some thoughts on how the future may change both in the EU and more widely afield.

24.2 The animal livestock industry and the origin of animal by-products (ABP)

The terrestrial livestock sector relies heavily on an essential and usually invisible industry operated by the animal by-products processers or renderers. In the past, renderers in particular have been termed the 'Invisible Industry', but more recently the industry has been described as 'Essential Rendering' (Meeker, 2006). However, in reality the processor of land or fishery by-products operates an important service on a truly global stage.

The modern ABP processing industry is illustrated diagrammatically in Fig. 24.1. Broadly, the basic principles are very simple. Raw animal or fish by-product contains high levels of moisture (~65% on average) and microbial load. The primary objective is to stabilise the raw material, by heating it to in excess of 80°C, to effect a microbiological inactivation. As a result, water is also evaporated, and as a further consequence, the fat is separated or 'rendered' from the other solid components. After a further physical separation such as centrifuging or expeller pressing, two products are produced: a high protein meal and a liquid fat. The terminology varies between the sectors. For rendering, rendered animal fat is usually called 'tallow' and the protein meal is termed either meat and bone meal (MBM) or processed animal protein (PAP). For fishery by-products, the protein is termed fishmeal and the fat is termed fish oil.

24.2.1 Rendering prior to the transmissible spongiform encephalopathy (TSE)/BSE crisis

In early years, rendering plants were in general smaller, mainly local to abattoirs and therefore centres of human population. What happened thereafter included the establishment of larger, more centralised rendering plants to a great extent, to take the benefits of economy of scale. In addition there was a switch from mainly batch processing to mainly continuous processing as new process and equipment developments were made. All of these processes were regulated to a certain extent by national regulators or authorities.

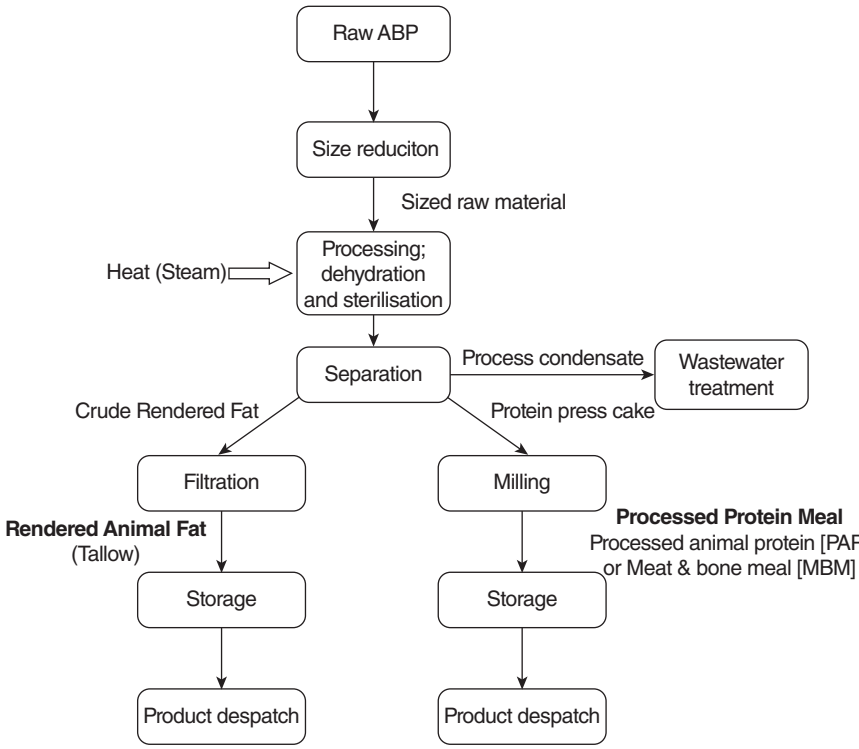

Fig. 24.1 Animal by-products: schematic process flow diagram.

However, in retrospect it is clear that not all standards in the European Union were equal or even equivalent. A first attempt to gain some sort of 'level playing field' in the EU was an agreement between Member States to publish the Animal Waste Directive which started the process of risk analysis and the establishment of process parameters. Subsequently, this directive was amended many times during the period 1990–2001, as new findings with regard to the safe processing of ABPs and the inactivation of TSEs were published. In contrast to these dramatic changes, the fish by-products industry has not altered dramatically over the last 20 years.

24.3 Rendering process evaluation

As a prelude to the actual TSE inactivation tests described below, a series of process evaluations were completed to confirm the real process parameters for a range of generic process systems to be evaluated (Woodgate, 1994). This preliminary work was necessary to confirm the following for process systems having the characteristics of type (batch or continuous), pressure (atmospheric, above, vacuum), and fat level (de-fatted, natural fat, added

fat); also, the particle size of the raw material was confirmed for each system. Thereafter the following characteristics were determined for each process under consideration: the proportion of fat present; the minimum transit time under normal and maximum throughputs; and the minimum and maximum times during the transit temperature profile. For the latter parameters, it was first required to determine the dynamics of different rendering process systems. An insoluble marker was developed to be able to determine the minimum residence times in continuous rendering cookers. Manganese dioxide (MnO_2) was chosen, as it is heat stable and can be prepared into a suitable form (as a briquette) for introduction into the rendering process. Importantly, manganese is present in animal by-products at rather low levels and would therefore not interfere with the test itself. These residence time studies have subsequently found continued use in the validation of rendering process plants under the animal by-products regulations in Europe.

As an integral part of understanding and minimising TSE risk, there was a need to study the potential for rendering systems to inactivate TSE agent. Most of the information about processing parameters used in Europe today originates from research into the inactivation by rendering of BSE and scrapie (TSEs) conducted in the UK between 1990 and 1994. The inactivation studies were published in two separate papers, one dealing with BSE, the other with scrapie (Taylor *et al.*, 1995, 1997). The TSE inactivation studies reported by Taylor *et al.* were the source of the specific time and temperature conditions that have since been applied to the EU rendering systems.

24.4 The TSE/BSE crisis and its implications

24.4.1 The European approach

Prior to 1988 animal by-products of all types were mixed together and rendered, usually in a local rendering process plant, and all types of animal proteins and fats were potentially used in animal feeds. The main aspect of risk management in this period was one of salmonella control in the protein meal. This situation changed dramatically with the emergence of TSE/BSE. BSE (in bovines) is one member of the cluster of prion diseases denoted TSE, and distinguished from scrapie in sheep and goats. MBM in animal feeds have been associated with the emergence of BSE in cattle in the UK, as the most likely vector for the infectivity was the early epidemiology pointing in the direction of infected MBM being used in feed for bovines. This route of infection has not been seriously disputed. Subsequently it became accepted that there was a risk to humans from consuming meat from animals fed feed infected with BSE agent. However, in animals slaughtered for meat consumption, a different approach was being taken. A multiple approach to human safety was taken in the UK. Firstly, all cattle over a certain age (30 months) were slaughtered and disposed of outside the food chain. Secondly, all bovines under 30 months

of age and also all adult ovines slaughtered to produce meat for human consumption had certain risk organs removed from the food chain. These materials are termed specified risk materials (SRM) and include the brain, spinal cord, intestine and other organs which have a risk of containing TSE infectivity.

24.4.2 Global approaches

The main approaches to prevent spread of TSE diseases in non-European countries have been to prohibit the use of certain tissues for use in animal feeds, rather than prohibit them from being rendered *per se*. Canada enacted an 'enhanced feed ban' based upon a 'short list' of specified ruminant by-products in July 2007. Under the enhanced feed ban, producers can no longer feed any animal products containing SRM to livestock, and abattoirs must properly identify SRM to ensure that it is removed from the feed system. In addition, a permit from the Canadian Food Inspection Agency (CFIA) is required to handle, transport or dispose of cattle carcasses and certain cattle tissues. This system enables continuous control over SRM, so that it does not enter the animal feed system. Specified risk material include the skull, brain, trigeminal ganglia (nerves attached to the brain), eyes, tonsils, spinal cord, and dorsal root ganglia (nerves attached to the spinal cord) of cattle aged 30 months or older, and the distal ileum (portion of the small intestine) of cattle of all ages.

The USA enacted an animal feed ban based upon a 'short list' of older dead animals and some specified ruminant by-products in April 2010. The US Food and Drug Administration (FDA) amended the agency's regulations to prohibit the use of certain cattle origin materials in the food or feed of all animals. This rule was effective from 27 April, 2009. These materials include the following: the entire carcass of bovine spongiform encephalopathy (BSE)-positive cattle; the brains and spinal cords from cattle 30 months of age and older; the entire carcasses of cattle not inspected and passed for human consumption that are 30 months of age or older from which brains and spinal cords were not removed; tallow that is derived from BSE-positive cattle; tallow that is derived from other materials prohibited by this rule that contains more than 0.15% insoluble impurities; and mechanically separated beef that is derived from the materials prohibited by this rule.

24.5 Processing of animal by-products

24.5.1 Animal by-products and categorisation

The key first step in the EU regulations relates to categorisation of the animal by-products. This is accomplished in accordance with the risk of these by-products to animals and humans. The key aspect of the categorisation relates to the risk assessments described in Section 24.4.1. The three categories are shown in Table 24.1.

Table 24.1 Summary of EU categories of animal by-products

Category	Animal by-products per category (non-exhaustive)
1	• Animals suspected of being infected by a TSE • Specified risk material, and entire bodies of dead animals containing specified risk material • Products of animal origin containing residues of environmental contaminants such as PCBs and dioxins if such residues exceed the permitted level laid down by Community legislation Mixtures of Category 1 material with either Category 2 material or Category 3 material
2	• Products of animal origin containing residues of veterinary drugs and contaminants if such residues exceed the permitted level laid down by Community legislation • Animals and parts of animals that die other than by being slaughtered for human consumption, including animals killed to eradicate an epizootic disease Mixtures of Category 2 material with Category 3 material
3	• Animal by-products or parts of slaughtered animals which are fit for human consumption in accordance with Community legislation, but are not intended for human consumption for commercial reasons • Parts of slaughtered animals which are rejected as unfit for human consumption but are not affected by any signs of diseases communicable to humans or animals and derive from carcasses that are fit for human consumption in accordance with Community legislation

24.5.2 ABP processing methods

The processing methods used for both land and fishery by-products in Europe are actually just a reflection of the global industry systems, as there are no unique systems used in one part of the world and not in others. Although practical variations between the basic processing methods do occur, both from one continent to another and also between companies within the same country, the key criteria for describing different rendering systems are as follows:

- Batch or continuous
- Natural fat, de-fatted, added fat
- Atmospheric, below atmospheric pressure ('vacuum'), above atmospheric pressure ('pressure').

24.5.3 Validation and safety of processing methods

The principles of rendering validation are based upon the experimental protocols for BSE and scrapie inactivation described in Woodgate (1994). There are seven systems currently approved for the processing of land

animal and fishery by-products. Of these systems, methods 1, 2, 3, 4 and 5 were set up to meet the requirements of maximum TSE inactivation (from the processing criteria that were established as a result of specific TSE inactivation experiments described above). Other methods were developed to meet exacting microbiological standards. One method (Method 6) is specifically approved for the processing of fish by-products, using a special hydrolysis method only, and a further method (Method 7) is approved for any process that is able to meet very stringent microbiological clearance standards.

24.5.4 Process evaluation and risk reduction

Rendering processes in Europe are approved in accordance with strict hygiene principles which use HACCP systems to deliver the objectives of producing safe products for a wide variety of uses. The principles of HACCP systems are, of course, well known and will not be repeated here. What is not so clear is how some of the critical control points (CCPs) are either established or applied. This section discusses the main elements that contribute to the HACCP-based approval regime laid down in the EU Animal By-Products Regulation (ABPR).

Residence time

With continuous systems, residence time is affected by raw material feed rate (assuming that fat recycle level is constant). The information gained here will be applied again later on, but from a practical point of view the operator will want to choose the maximum feed rate for his plant that can meet the validation criteria. Here is needed a period of trial and error to ascertain the optimum conditions.

Temperature

Accurately measuring temperature in continuous cookers has been a challenge for engineers. Much of the early work has again involved trial and error. Eventually, it was decided to use thermocouples, housed in sleeves introduced through the wall of the cooker. The design of these units was such that the contact with the temperature of the material being processed was maximised whilst minimising the possibility of physical damage or the effect of the steam jacket on the temperature. To achieve the temperature profile information in the correct format, the thermocouples are placed at positions along the length of the cooker that can be recorded as percentage flow along the cooker, starting with the entrance at 0% and the exit at 100%. Ideally five thermocouples are employed, one close to the entry point, one close to the exit and the other three spaced along the cooker. Normally the thermocouples are linked to a data recorder, so continuous measurement records can be kept of both the validation tests and subsequent day-to-day operations as part of the HACCP system. In

the actual validation test period, actual temperature values are used to determine the operating criteria.

Process approval
After the preliminary work has been completed the process can be validated to operate at or above the approval standards. The process should be operating at steady state during the test according to the desired conditions of particle size, raw material feed rate, fat recycle rate and steam pressures to achieve the required temperatures. The key test on the day is the marker flow rate test. The marker test is completed under typical operating conditions which are all recorded for use afterwards. The data recorded is then applied when the results of the manganese marker trials are able to be plotted against time. A typical validation approval issued by the competent authority shows the validation key points as CCPs, with some of the parameters, such as raw material feed rate or fat addition level, described in practical terms, such as pump settings and fat level control respectively.

In addition to the physical validation criteria (CCPs) microbiological samples are taken at the time of the validation test and at other times in accordance with EU rules. Each of these microbiological species is determined on samples produced on an ongoing basis (normally hygiene samples taken 'in house' and official samples taken from dispatch loads).

Conclusions
It is possible to validate a rendering process according to the procedure described, and to lay down the CCPs to ensure that the specific validation conditions are met. Following the validation and approval, operation of the plant according to HACCP principles should ensure that the approval standards are continuously met. Revalidations should, in practice, only be required if or when there are significant changes to the process such as a replacement or new cooker unit or a redesign of the plant layout.

Of course, when using a HACCP system, it is necessary to lay down the corrective actions following any breach of a CCP. Non-compliances and corrective actions for each CCP are shown in Table 24.2. Non-compliant PAP/MBM produced may be subject to recall and reprocessing.

24.5.5 Current legislation pertaining to ABP

The current and main legislative instrument for both land and fishery by-product processing in Europe is called the Animal By-Products Regulation (ABPR) (1069/2009). It is supplemented by an implementing Regulation (ABPR-IR) 142/2011. The primary regulation is very extensive and the full title conveys the level of interest and input into the framing of this piece of legislation by both the public (European Parliament) and EU Member State governments (Council). The implementing regulation lays down all of

Table 24.2 Non-compliance and corrective action for CCPs

CCP	Non-compliance and corrective action
1	Place any worn anvils or breakers to ensure minimum particle size. Re-process PAP/MBM produced under non-compliant conditions.
2	Ensure maximum feed rate is not exceeded. (In practice, control with safety margin.) Re-process PAP/MBM produced under non-compliant conditions.
3	Ensure maximum fat addition rate is not exceeded. (In practice, control with safety margin.) Re-process PAP/MBM produced under non-compliant conditions.
4	Ensure that exit temperature does not fall below minimum temperature. Establish 'action zone'. Stop raw material feed from entering action zone, and allow temperature to rise before recommencing raw material feed. Re-process PAP/MBM produced under non-compliant conditions.

the technical requirements of the primary regulation and both are required to be understood and complied with to meet the full requirements.

ABPR/1069 includes all of the key aspects regarding categorisation, processing, disposal and use of all animal by-products. The key aspects of the new regulation (1069/2009) are very similar to those described in the predecessor of the current regulation (ABPR/1774) and are described in Woodgate and van der Veen (2004).

In respect of materials produced for animal feeds, both (parts of) the ABPR/1069 and ABPR-IR/142 and another regulation, known as the transmissible spongiform encephalopathies (TSE) Regulation (TSER 999/2001), need to be considered together. ABPR/1069 contains important elements regarding the categorisation of raw materials suitable for producing animal feed-grade products.

The use of certain products as ingredients for feed for animals used in food production for humans is in principle approved subject to conditions:

- Category 3 is the only category able to be used in the foodchain.
- There are strict processing conditions laid down.
- There is a prohibition on intra-species recycling.

When considering animal products as ingredients for animal feeds, the TSER is both important and directly relevant. Interestingly it is the TSER that actually defines what is by meant an animal; i.e. one that is used for producing food for humans. This definition is in contrast to domesticated companion animals and farmed fur animals for which other regulations may apply. The mechanism within the TSER is that it firstly prohibits *all* animal proteins for use in animal feeds and then (in Annex IV) makes exceptions for those that are allowed to be used. Here any restrictions on use, such as to certain species only, are cited.

It is important to note that there are no restrictions on the use of rendered animal fats, save the one condition (in the ABPR/ABPR-IR and the TSER) that requires tallow derived from ruminants to have a maximum of 0.15% of insoluble impurities if it is to be used in animal feeds.

There are compliance microbiological standards for production of animal products that are intended for use in animal feeds. In the EU three sentinel organisms are used, each having a place within the process and regulatory HACCP system.

Products for use in animal feeds are required to meet the following:

- *Clostridium perfringens*: Absent in 1 g
- S*almonella* sp: Absent in 50 g
- Enterobacteriaceae: Samples must meet specific limits.

24.6 Risk identification and management in animal feeds

Some risk analyses for the production chain for feed materials manufactured from the following materials have been developed that build upon HACCP principles and consider all of the potential risks to the food chain. These risk analyses list the safety hazards that may occur throughout the production chains, up to the delivery of the feed material to the feed compounding industry. This work has been completed by industry collaboration with the European Feed Ingredients Platform (EFIP) in relation to the proposed EU scheme known as the European Feed Ingredients Safety Certification (EFISC). The risk analyses indicate the level of risk that each hazard will pose to feed safety and whether that is to be controlled by a measure as part of a pre-requisite programme (PRP) or by a critical control point (CCP). The risk analyses list applicable legal trade limits and formulate necessary measures that need to be taken to reduce the hazards to acceptable levels.

One of the key areas of legislative control on animal feeds is in the arena of dioxins, dioxin-like PCBs and non-dioxin-like PCBs. Here in Europe there are maximum limits set for feed ingredients and finished feeds. There are also action limits set, at which actions should be taken to prevent the likelihood of contamination of finished feed at critical and dangerous levels (for details see also Chapter 8).

24.6.1 Feed contamination with MBM

In the EU there are extremely onerous regulatory controls in place to control the safety of animal feeds. The feed ban, discussed above, effectively requires that samples of finished feed are tested to ensure that they do not contain any animal proteins. The natural presence of bones in nearly all types of animal proteins used in animal feeds affords the possibility of using

the bones present as a marker to indicate the presence of animal proteins in finished animal feeds. The policy of 'zero tolerance' in respect of the presence of MBM in animal feeds adopted in the wake of the BSE epidemics meant that effectively if just one 'terrestrial' animal bone was found in the sample of feed, then it was deemed to be contaminated with MBM. This very simplistic approach was probably the most practical way forward at the time, but the zero tolerance soon led to several different and unforeseen consequences (details on the methodologies applied in the detection of animal by-products are presented in Chapter 6).

24.6.2 Current EU status and initiatives

Details of the EU TSE regulation may change according to the procedures laid down in the EU legislation. This approach involves all of the EU 27 Member States, in the form of the Standing Committee on the Foodchain and Animal Health (SCoFCAH), approving proposed amendments. Any of the recent amendments are almost certain to have been subject to risk assessment beforehand. Therefore, any successful amendment to what is a very strictly limited list of approved animal feed ingredients, will have been subjected to a three-tier level of approval, i.e. EFSA, the Commission, the Member States. What is more, the European Parliament also has a right of scrutiny and interjection if it so wishes. Table 24.3 summarises the materials that are currently (2010) prohibited and authorised in animal feeds in the EU.

There have been and continue to be a range of initiatives in play in Europe to try to achieve the goal of more approvals for processed animal proteins. In the EU there are several layers of regulatory controls in place that form an overarching approach to controlling the safety of animal feeds. The key tool is the official method for detection of bone particles in animal feeds. This method was adopted in the very early days of the (animal) feed ban and in principle was a method of detecting meat and bone meal (MBM) in compound animal feed. It is important to note that the method is validated in respect of terrestrial bones and is able to differentiate land animal bones from fish bones. This is important, as fish meal (also a processed animal protein according to the ABPR) was not banned for use in pig and poultry feeds. One of these consequences was that even feathermeal was prohibited as it too contains (poultry) bones and this could not be differentiated from any other bones that might have been present if MBM was included in the animal feed. On the other hand, vegetable root crops such as sugar beet were found to contain mammalian bone fragments from time to time. This situation was certainly unforeseen and on investigation was found to be related to contamination in the growing fields from the bones of dead mice or birds. This latter finding did lead to a change in legislation: an aspect of tolerance linked to a risk assessment was introduced to achieve a pragmatic solution that did not stop beet harvesting if just one

Table 24.3 Summary of EU feed legislation: prohibited and approved materials

	Prohibited		Authorised	
	Permanent ban	Temporary ban	With restrictions	Completely
Ruminants	• Animal protein and feeding stuffs containing mammalian protein (PAPs, blood products, etc.) • Feeding stuffs containing PAPs or DCP and TCP of animal origin		• Fishmeal for unweaned ruminants (from 2008, conditions of use)	• Milk, milk-based products and colostrum • Eggs and egg products • Animal fat • Hydrolysed proteins derived from parts of non-ruminants and from ruminant hides and skins, feathers and fish • Gelatine from non-ruminants
Non-ruminant farm animals (pig and poultry)		• Non-ruminant PAPs • Proteins of ruminant origin (gelatine, blood, PAPs)	• Fishmeal (conditions of use) • DCP and TCP (conditions of use) • Non-ruminant blood products (conditions of use) • Animal fats (of ruminant origin with special processing)	• Milk, milk-based products and colostrum • Eggs and egg products • Animal fat • Gelatine derived from non-ruminants • Hydrolysed proteins derived from parts of non-ruminants and from ruminant hides and skins
Fish			• Non-ruminant blood meal and blood products (conditions of use) • Fishmeal (conditions of use) • DCP, TCP of animal origin (conditions of use)	• Milk, milk-based products and colostrum • Eggs and egg products

mammalian bone fragment was found in a consignment. Nonetheless, the issue of 'tolerance' has not yet been fully agreed either in respect of (cross) contamination of one animal protein in another PAP or in terms of species-pure PAP in finished or compound feeds.

The approach of the European Commission with regard to risk in animal feed is based upon the avoidance of 'ruminant protein' (of any category 1, 2, 3) in any farmed animal feed, to avoid any chance of it being ingested by ruminants. Therefore many of the control tools concentrate on the detection of ruminant protein at low levels both in PAP and in animal feeds. The 'tolerance' level in each is expected to be different, and the proposals under consideration are discussed accordingly. On a more market-oriented note, EFPRA have decided to term the PAP made from pigs and poultry (both monogastric animals) monoPAP in order to differentiate it from other types of PAP, such as ruminant PAP (used in petfoods) and fishmeal (fishPAP).

Several key initiatives have been taken to try to achieve the following objectives:

- Differentiate Category 3 PAP from Category 1 or 2 protein meals using a marker.
- Determine the risk (by risk assessment) of additional TSE cases in Europe by the cross-contamination of animal feeds with ruminant protein.
- Develop a method for species detection suitable to detect ruminant proteins in non-ruminant PAPs, with appropriate tolerances.
- Develop an enhanced system that delivers a secure supply of species-pure PAP: from the source of the Category 3 ABP to the delivery of the PAP to the feed mill and farm.
- Establish a new official method in Europe for the detection of ruminant proteins in finished or compound feeds.
- Work with all stakeholders in the 'foodchain' to develop a strategy that meets the objectives of all members.

In practice, this work has progressed over the last five years with a range of projects and studies being completed by both industry and official laboratories. Importantly, in this period, the European Commission has established a central laboratory of expertise in the subject of animal proteins. The Community Reference Laboratory – Animal Proteins (CRL-AP) is based in Gembloux, Belgium, and has been involved since 2007 with many aspects of the animal protein work.

Markers

This work involving EFPRA and the EU Joint Research Centre (JRC) in Geel, Belgium, developed and validated a marker, glycerol triheptanoate (GTH), for addition to Category 1 and 2 processing plants.

Risk assessment

EFPRA commissioned an independent report on the potential risk from cross-contamination of ruminant protein by DNV, a worldwide risk assessment company. The model used was the same one used by the European Food Safety Authority (EFSA) when calculating the risk of cross-contamination by MBM in animal feeds. The EFPRA/DNV Risk Report results indicate that if there is a limit of detection as high as 5% ruminant protein in monoPAP (together with a limit of detection of 1% monoPAP in ruminant feed), and that these levels applied to all of the cattle feed produced in the EU, then the risk of additional BSE cases would be extremely low and significantly lower than the value reported in the EFSA's 'Opinion of the Scientific Panel on Biological Hazards on the quantitative risk assessment of the animal BSE risk posed by meat and bonemeal with respect to the residual BSE risk' (EFSA, 2005). It is also noteworthy that the European Commission have updated the 'TSE Roadmap' and have published a TSE Roadmap 2 (2010) with a view to the possible adaptation of certain measures of the current feed ban.

PAP species identification

This work has been conducted by EFPRA/CCL in conjunction with the CRL-AP. The outcome has been a development of an ELISA-based method (Melisatek™) that is able to detect 1% ruminant protein (processed up to 137°C under pressure) in a mixture with non-ruminant proteins at a sensitivity of 100%.

LYNXX: a secure supply chain

This system is still under development, but is considered to be important in bringing confidence into the supply of monoPAP for animal feeds. One of the main aspects of LYNXX is the requirement for segregation at all parts of the chain, beginning with slaughterhouses, then process (rendering) plants, and finally feed mills. Specific transport codes of practice will also apply at the appropriate stages.

Official methods

Development and validation of methods is the responsibility of the CRL-AP and the European Commission. Two major EU projects have assisted in this process:

- EU R&D project 'STRATFEED': Objective to improve and if possible quantify official microscopy method for animal feeds and to develop other species detection methods.
- EU R&D project 'SAFEED-PAP': Objective to continue developments above and to develop and validate a suitable method that enhances the official microscopy method.

It now appears that a new official method may be validated within the next nine months or so. At least two PCR methods for detecting ruminant proteins in finished feed at a level of 0.1% are under consideration to be included in an inter-laboratory study (ring trial) to be completed in 2011. If successful, one or more PCR methods could be published as new 'official EU methods'.

Stakeholders
EFPRA are members of a group bringing together different individual sector associations: farmers, red meat, poultry meat, meat processors and animal feeds. This group have come together to work on both the technical and political aspects of gaining the authorisation of monoPAP for use in animal feeds.

24.6.3 EU rapid alert

In Europe there is a system in place termed the 'Rapid Alert System for Food and Feed'. It has been established in response to 'crises' in the food chain that have occurred in the last decade. Apart from BSE (which as an epidemic would probably not have been prevented by such a system), the main initiator was the Belgian dioxin crisis. The rapid alert system is organised to try to prevent any future crises, and is operated effectively by national competent authorities that are required to notify the Commission of their own findings. As a result, the information is publicly available as soon as possible, so that all involved in the chain may be able to take some actions.

Some examples appearing in the rapid alert reports are microbiological contamination, bone particles, chemical contaminants such as dioxin/PCBs or natural toxins (mycotoxins and others). None of these contaminants should, in principle, be present in quality feed products, but if mistakes occur and these contaminants do find their way past the first safety net, then this system can be regarded as a further safeguard.

24.6.4 Codex Alimentarius

Globally, all feed regulation requirements should conform with the Codex Alimentarius Code of Practice on Good Animal Feeding (CAC/RCP 54-2004). This Codex Code has been converted to a Feed Manual, published jointly by FAO and IFIF: *Good Practices for the Animal Feed Industry*.

Currently, there is still a worldwide ban on the use of ruminant proteins in ruminant feeds. This position, first proposed in UK and then in Europe, has become the norm for ruminants. However, in many parts of the world ruminants are allowed to be fed non-ruminant animal proteins and animal fats from all species, including ruminant-derived fat.

As described in Section 24.4.2, Canada and the USA have enacted 'enhanced feed bans' based upon the removal of certain high-risk raw materials from the feed and food chain.

24.7 Future trends

The European approach of ultra-precaution has been rigidly applied for about 10 years, so 2012 and 2013 are important years in Europe. There is high expectation that monoPAP will be authorised for aquafeeds in 2012 or 2013, and for pigs and poultry soon after. The demand for monoPAP is high, both inside and outside Europe, but as a prerequisite for export from Europe for use in animal feeds, the monoPAP must be legally saleable within the EU. Now with the technical solutions on the horizon and a high demand for the supply of animal-derived nutrients, the future for the European animal by-products sector looks promising.

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24.9 Appendix: glossary of terms

EU Food Regulations (853/2004)
The EU Food Regulations (of which this is one of a series of five) lay down hygiene and processing conditions for certain edible co-products such as carcase fat and bones.

EU Animal By-products Regulation (ABPR/1774 as amended)
The EU Animal By-products Regulation 1774/2002 lays down health rules concerning animal by-products not intended for human consumption.

EU Animal By-products Regulation (ABPR/1069)
The EU Animal By-products Regulation 1069/2009 lays down health rules concerning animal by-products not intended for human consumption.

EU Animal By-products Implementing Regulation (ABPR-IR/142)
The EU Animal By-products Implementing Regulation 142/2011.

EU TSE Regulation (999/2001 as amended)
The EU TSE regulation (TSER) lays down specific details regarding removal of Specified Risk Materials (SRM) from bovines and ovines, and age limits for BSE testing. The TSER also contains a list of non-approved and approved derived products that may be used in feeds for farmed animals.

Edible co-products
Materials that are processed in accordance with the food regulations, from which derived products may be used in animal feed. The process used is termed melting or edible rendering.

Category 3 animal by-products
Category 3 animal by-products comprise exclusively animal by-products derived from approved animals. These approved animals are slaughtered in an approved slaughterhouse and are considered as fit for human consumption after an ante- and post-mortem veterinary inspection in accordance with the Community legislation. The process used is termed rendering.

Derived products
Products derived from processing of certain edible materials or animal by-products in accordance with the EU Food, ABPR and TSE regulations.

Rendered animal fats
Animal fats derived from edible co-products or Category 3 animal by-products by melting/rendering.

Processed Animal Proteins (PAP)
Animal proteins derived from edible co-products or Category 3 animal by-products by melting/rendering.

Approved products
Blood products, gelatin, hydrolysed proteins and di-calcium phosphate are approved products in the ABPR/1774. However, the TSER/999 currently approves the above for use in animal feed and also approves the use of non-ruminant blood meal for use in aqua-feeds only.

Feed ingredient
Any derived product used in feed for animals farmed for food production.

Animal feed
Feed for animals farmed for food production, including terrestrial and aquatic animals.

Petfood
Feed for pet species normally nourished and kept as companions, but not consumed by humans.

Fur animal feed
Feed for farmed fox or mink species, not consumed by humans.

STRATFEED
An EU research project on 'Strategies and methods to detect and quantify mammalian tissues in feedingstuffs'.

SAFEED-PAP
An EU project on 'Species-specific detection of processed animal proteins in animal feed'.

TSE Roadmap
A Communication to the European Parliament and the Council which outlines areas where future possible changes to EU legislation on TSEs could be made.

25

Management of animal feed safety in the USA

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Abstract: The US regulatory system for animal feed and pet food safety is made up of programs and policies addressing specific, identified hazards. In general, the system has worked well, but challenges have come from unexpected sources. The Food and Drug Administration has established the Animal Feed Safety System (AFSS) Initiative to analyze the feed safety regulatory system and developed recommendations to address 'gaps' in the current system. Meanwhile, the melamine contamination crisis highlighted the evolving nature of international commerce in food and animal feed, and the need for a review of the policies used to protect animals and humans against feed-borne risks. And, in 2011, the Food Safety Modernization Act became law, bringing additional changes to the rules applying to feed safety.

Key words/phrases: animal feed and pet food safety, regulation of animal feeds, pet foods, and ingredients, animal feed safety system, current Good Manufacturing Practice regulations (cGMP), FDA-licensed and non-licensed feed mills, Food Safety Modernization Act (FSMA).

25.1 Introduction

In the United States, the basic law regarding the safety of food is the Federal Food, Drug, and Cosmetic Act, and the definition of 'food' in that Act encompasses feed for animals and food for pets as well as food for human consumption. Section 201 of the Act states, 'The term "food" means ... articles used for food or drink for man or other animals ... , and ... articles used for components of any such article.'[1] As well as defining food, the Act also provides the authority primarily to the US Food and Drug Administration (FDA) to regulate food for man and animals. Within FDA, most of the regulatory work concerning animal feed and pet food safety is done by the Center for Veterinary Medicine (CVM).

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The regulatory system FDA/CVM has developed over the years is made up of programs and policies addressing specific, identified hazards. For example, CVM developed 'current Good Manufacturing Practice' (cGMP) regulations as a mechanism to ensure the safety of medicated feed and regulations for preventing the establishment and spread of bovine spongiform encephalopathy (BSE) in the United States. In the area of feed contaminants, CVM has a regulation that calls for no *Salmonella* in certain animal-based feed ingredients and policies for limiting mycotoxins in feed ingredients.

Overall, the feed safety system has worked well in the United States, but challenges have come from unexpected sources, such as dioxins or melamine in feed ingredients. Partly because of unexpected challenges to the safety of feed, but more due to the fact that weaknesses in the regulatory system were being identified, FDA began a review of the elements of the feed safety system in the United States.

Beginning in 2003, FDA established the Animal Feed Safety System (AFSS) Initiative. Under the Initiative, a team assembled to carry out the AFSS Initiative began an analysis of the entire regulatory feed safety system in the United States and developed recommendations to address various 'gaps' in the regulatory coverage of the current system. The work being done under the AFSS fits well within the newer food safety laws, programs, and policies developed in 2007 and later, as the United States addressed the crisis created by the discovery of melamine contamination of imported feed ingredients that were used to make pet food and in some cases ended up in animal feed.

The melamine contamination crisis highlighted the evolving nature of international commerce in food, including animal feed and pet food, and the need for a review of the policies used to protect against food and feed risks. The crisis highlighted the amounts of food and feed ingredients traded internationally and how broadly throughout the food chain imported products are distributed. (The discovery of melamine contamination resulted in recalls by 100 companies of more than 60 million servings of pet food; feed from hog farms in eight States, and from 30 broiler farms and eight breeder poultry farms.[2]) As a result of the heightened awareness of the need for increased food and feed safety protection, the US Congress passed legislation in 2007 to give FDA more mechanisms to keep pet food and animal feed safe. The crisis also prompted regulatory officials in the US government to review the approach to food safety used in the United States.

25.2 Specific feed safety programs

FDA has had responsibility for *feed* safety since it took on the responsibility for *food* safety under the original Pure Food and Drug Act of 1906. But the

role of the Federal government in feed safety increased with the feed industry's use of medicated feeds in the mid-1960s. One of the oldest feed safety programs is the program of the current Good Manufacturing Practices (cGMPs) regulations.

25.2.1 cGMP

The cGMP program for medicated feed was first implemented in 1965. Over the years, the program has been updated. As the program is now configured, those medicated feed manufacturing facilities using the most potent form of certain animal drugs must have FDA licenses, operate under more stringent cGMPs, and be subject to biennial inspections to ensure compliance with the cGMPs and related other regulations.

FDA classifies drugs used in feeds in two categories. Category I drugs require no withdrawal time. (A withdrawal time is the time between when an animal is last given an animal drug, for instance in feed, and when the animal or products from it can be marketed for food.) For Category II drugs, FDA either requires a withdrawal time or has established a zero tolerance for the residue limit of the drug in edible tissue.

Those two categories are further divided by types. Type A medicated articles contain the most concentrated form of the drug and cannot be used for feed. Type A medicated articles must be further mixed with feed ingredients to produce Type B or Type C medicated feed.

Feed manufacturing facilities using Category II Type A medicated articles must be licensed by FDA. Other medicated feed manufacturers do not need FDA licenses.[3]

FDA uses two sets of cGMP requirements for medicated feed manufacturing facilities. The cGMPs requirements that apply to FDA-licensed feed manufacturers[4] are a more stringent set than those that apply to non-FDA licensed manufacturers.[5] FDA-licensed medicated feed manufacturing facilities are subject to an inspection every two years to determine whether they are meeting the cGMP requirements. Feed mills that do not require a license are not subject to the same biennial inspection requirement, but can be inspected to determine compliance with the applicable cGMP standards at any time.

The cGMP regulations address issues such as the capabilities of personnel employed at the facility; what constitutes appropriate facilities and equipment; product quality control and quality assurance; packaging and labeling; records and reports; and customer complaints.

The cGMP regulations are not meant to give precise instructions to feed manufacturers about the processes they should use. In practice, the cGMP regulations describe the minimum requirements that a feed manufacturer must meet when producing a medicated feed in order to be in compliance with the regulation. Manufacturers who are in compliance are considered capable of producing a medicated feed that would not be considered

adulterated. FDA wrote flexibility into the regulations because of the wide diversity in the types of feed manufacturing facilities that fall under the cGMP requirements. Additionally, the flexibility allows for equipment and facility changes resulting from innovation and modernization.

The differences in cGMP requirements for FDA-licensed and for non-licensed facilities are clearly seen in the labeling standards. The cGMP regulations for FDA-licensed feed manufacturing facilities include requirements for handling the labels, for proofreading them when they are returned from the printer (with the requirement that the party responsible for proofing the labels initial a record for the file and that the proofed label is kept for one year), for making sure the correct labeling accompanies shipments of bulk feed, and for periodically reviewing label stock to make sure discontinued labels are discarded.

By contrast, the cGMP for non-FDA licensed medicated feed manufacturers says only:

> 'Labels shall be received, handled, and stored in a manner that prevents label mixups and [ensures] that the correct labels are used for the medicated feed. All deliveries of medicated feeds, whether bagged or in bulk, shall be adequately labeled to [ensure] that the feed can be properly used.'

Regardless of whether medicated feed was produced in an FDA-licensed or a non-licensed feed mill, FDA considers medicated feed to be adulterated if it was produced in a facility that does not meet applicable cGMPs standards.

25.2.2 Sampling for *Salmonella*

Under section 402(a) of the Federal Food, Drug, and Cosmetic Act, *Salmonella*-contamination causes feed, feed ingredients, or pet food to be considered adulterated. By regulation, animal byproducts intended for use in animal feed (bone meal, blood meal, crab meal, feather meal, fish meal, fish solubles, meat scraps, poultry meat meal, tankage, or other similar animal byproduct, or blended mixtures of these) will be regarded as adulterated and unacceptable for use in feed if they are contaminated with *Salmonella* bacteria.[6]

FDA policy is that feeds must be '*Salmonella* negative,' which is determined by taking 10 100-gram samples or more for testing. If *Salmonella* is not found in any of the samples, the feed is considered to be *Salmonella* negative.

The greatest concern for public safety is pet food, treats, and supplements that consumers directly contact. Pet foods are intended to be fed to pets in homes where they are likely to be directly handled by humans. *Salmonella*-contaminated pet foods can pose a significant health risk to humans, especially children, the elderly, and individuals with a compromised immune system. FDA's regulatory focus is aimed at keeping

Salmonella-contaminated pet food out of interstate commerce. FDA has routinely conducted a sampling program of pet foods to detect *Salmonella*.

According to the sampling program, CVM 'is concerned about animal feeds serving as vehicles for transmitting pathogenic and antibiotic-resistant bacteria to humans and other animals and is particularly concerned about *Salmonella* being transmitted to humans through direct-human-contact feeds. *Salmonella*-contaminated feeds pose a significant health risk to humans who contact or ingest them, as well as to animals that consume these feeds.'[7]

25.2.3 Limiting mycotoxins

The Federal Food, Drug, and Cosmetic Act says that food or feed is considered adulterated if it contains any poisonous or deleterious substance that renders it injurious to health. One class of poisonous or deleterious substance that is commonly found in food and feed is called 'mycotoxins'. Mycotoxins are chemical substances produced by molds (fungi) that grow on food and feed products. In some cases, mycotoxins can cause feed to be considered adulterated. Different mycotoxins have different deleterious effects, and those effects will vary depending on animal species or classes. FDA decides on a case-by-case basis what level of mycotoxins will make feed injurious. FDA focuses on the following five major mycotoxins.

Aflatoxins

The FDA Aflatoxin Action Levels is for the total aflatoxins (B1 + B2 + G1 + G2). FDA feed safety experts believe that all aflatoxins have similar toxicity. They have the similar chemical structure and same mode of action. FDA has established 'action levels' for aflatoxins. If aflatoxins are found in a feed or feed ingredient at levels that exceed an action level, FDA could declare the feed or ingredient adulterated. However, action levels are non-binding on FDA, the industry, and the courts. Therefore, each time FDA takes regulatory action against a product because of aflatoxin content, the Agency must prove the product is unsafe.

FDA has established no single action level for aflatoxins. The action levels are different for different ingredients, and they vary depending on which animal will receive the feed. For instance, the action level for the total aflatoxins (B1 + B2 + G1 + G2) for corn and peanut products intended for finishing (feedlot) beef cattle is set at 300 parts per billion (ppb). The total aflatoxin action level for corn, peanut products, cottonseed meal, and other animal feeds and feed ingredients intended for dairy animals is 20 ppb. (A list of all action levels is available in CPG Sec. 683.100 'Action Levels for Aflatoxins in Animal Feeds.')

Fumonisins

FDA has established guidance levels for fumonisins, for both human food and animal feed. The levels are what FDA considers to be adequate to protect human or animal health and are 'achievable ... with the use of good agricultural and good manufacturing practices,' FDA said in its Guidance for Industry document.[8] The guidance levels are based on total levels of fumonisins (FB_1 + FB_2 + FB_3) in corn and corn byproducts intended for animal feed, and the guidance specifies that corn and corn byproducts containing these levels should be used for only 50% of the diet. The guidance level for corn and corn byproducts for poultry being raised for slaughter is 100 parts per million (ppm), for ruminants three months of age or older being raised for slaughter is 60 ppm, and for swine is 20 ppm. Like action levels for aflatoxins, guidance levels for fumonisins are not binding on FDA, the industry, or the courts.

Vomitoxins

FDA has issued advisory levels for vomitoxin (DON) in livestock feed. They are:

- For chickens – grain and grain byproducts should not exceed 10 ppm and should not be used for more than 50% of the diet. The finished feed should contain no more than 5 ppm vomitoxin.
- Swine – grain and grain byproducts should not exceed 5 ppm, and should not be used for more than 20% of the diet. The finished feed should contain no more than 1 ppm vomitoxin.
- For ruminating beef and feedlot cattle older than four months, and for ruminating dairy cattle older than four months – grain and grain by products (on an 88% dry matter basis) should not exceed 10 ppm, and distillers' grains, brewers' grains, and gluten feeds derived from grains (on an 88% dry matter basis) should not exceed 30 ppm. The finished feed should not exceed 10 ppm for ruminating feedlot and beef cattle over four months of age.

The other two mycotoxins are ochratoxin A and zearalenone. FDA has not established an action, advisory, or guidance level for these two mycotoxins.[9]

25.2.4 Bovine spongiform encephalopathy (BSE)

To address concerns about the establishment and spread of BSE in the United States, CVM developed a rule to prevent the transmission of the infectious agent through feed. The rule, implemented in 1997, prohibits the use of most mammalian tissue in feed for cattle and other ruminants. Enforcement is done through inspections. When the rule was first implemented, Federal and State inspectors gave highest priority to inspections

of rendering facilities and commercial (off-farm) feed manufacturers. The compliance plan was to inspect 100% of these firms.[10]

In 2008, FDA implemented an additional rule that prohibits the use in any feed, including pet food, of specified material that is known to have the highest risk of carrying the agent that causes BSE. The prohibited material includes the brains and spinal cords of cattle 30 months of age or older and the entire carcass of cattle older than 30 months of age if the brain and spinal cord have not been removed, and any part of a carcass of an animal diagnosed with BSE.[11] The goal of this rule is to prevent contamination – either inadvertently through mixing errors or cross-contamination, or deliberately – of ruminant feed with prohibited material deemed high risk.

25.3 The US Animal Feed Safety System (AFSS)

All of these regulatory approaches to feed safety have worked well, and the vast amounts of feed produced and consumed in the United States each year are safe. However, unanticipated problems have occasionally occurred.

Therefore, in 2003 FDA began the AFSS Initiative, and asked the AFSS Team members to perform a systematic review of feed safety rules and policies in place and identify 'gaps' in which either no process existed or the processes were inadequate to protect feed and needed to be modernized.

At an AFSS kickoff meeting in September 2003, the CVM Director at the time, Dr Stephen Sundlof, said:

> '*We've all heard about a lot of the issues, BSE being obviously the most prominent in recent times, but other things such as PCBs and dioxin continue to come up now and then, and dioxin is currently a hot topic. ...*'
>
> '*Currently, we've tended to deal with these issues one issue at a time, so we deal with the dioxin issue or we deal with the BSE issue or we deal with the* Salmonella *issue without really taking the broader look at (whether) we have a good overall system in place that ...would have more preventive effect. ...*'[12]

The AFSS Team is led by CVM and includes representatives from FDA's Office of the Commissioner and from State feed control officials. As it has conducted the review, the AFSS Team has been describing its progress in the 'Framework Document of the FDA Animal Feed Safety System,' which is posted on the FDA website.

The fourth version of the Framework Document states that, although the feed safety record in the United States is good, 'because oversight of this industry is limited and focused on a few known safety issues, potential human and animal health problems remain hidden.'

As part of its analysis, the AFSS Team has developed these principles of feed safety. The principles include:

- The feed and animal production industries are ultimately responsible for feed safety, and the government regulators can provide rules, guidance, and oversight to help the industry meet its feed safety goals. The regulatory oversight will give producers flexibility to meet safety criteria.
- AFSS extends to all parts of the feed production system, including ingredient suppliers and to transportation companies used to haul ingredients and finished feeds. In addition, the need to be aware of feed safety practices extends to actual use of the feed.
- The AFSS approach will incorporate risk-based decision making to determine the greatest hazards to humans or animals, so that enforcement resources are directed to the greatest risks.
- FDA will use its resources to address feed hazards that pose the greatest risk to humans and animals, and use the best methods for addressing them.
- FDA will engage in research to advance feed safety.

Under AFSS, FDA defines a hazard in feed as a biological, chemical, or physical agent in feed or a condition of feed that could cause illness or injury to animals or humans. The goal of the AFSS Team is to identify hazards that are actual risks. 'One challenge in drafting the AFSS Framework is defining terms in a way that takes into account the fact that the presence of certain agents in feed or existence of certain conditions of feed does not always possess a significant risk to animal or human health. It is when controls are not adequate at feed establishments that these same agents or conditions of feed may cause the feed to be a much greater risk to animal or human health,' according to the AFSS Framework Document.

An example cited in that document is aflatoxin contamination of corn for feed. Aflatoxin levels below 0.1 ppb are not likely to cause adverse health consequences – to the animals or to humans consuming food derived from those animals. However, if the contaminated corn is stored in such a way that aflatoxin levels climb to more than 20 ppb, a greater risk exists for animals and humans. In this case, what was a hazard – an agent or condition of feed with the potential to cause illness – can become an unacceptable feed risk likely to causes illness or injury to animals or humans.

The AFSS Team has analyzed the existing body of regulations and policies to identify where changes were needed. Here is a list of the AFSS-identified feed system regulatory components and some of the gaps the Team has identified. (For complete information about all the gaps, go to the AFSS page on the FDA website, http://www.fda.gov/AnimalVeterinary/SafetyHealth/AnimalFeedSafetySystemAFSS/default.htm, and click on the most recent draft of the 'Framework of the FDA Animal Feed Safety System.')

25.3.1 Safety of ingredients (component A)

In the United States, feed ingredients that are considered Generally Recognized as Safe (GRAS) or as food additives are specifically cited in FDA's regulations. The regulations do not provide a complete list of all the ingredients that may be considered GRAS for an intended use in animal feeds. The Federal Food Drug and Cosmetic Act also provides that an individual may make a determination that the use of a substance is GRAS. Using the GRAS notification program, an individual may notify the FDA of his or her decision that an ingredient is GRAS for a certain intended use. These notifications will be found on the FDA web page. All other ingredients that FDA considers acceptable for safe use in feed are not listed in any official government publication, because the Agency does not have an official listing of acceptable ingredients. The Association of American Feed Control Officials (AAFCO), a group made up of representatives from authorities in each State, Puerto Rico, Canada, and Costa Rica, maintains the best list of ingredients suitable for use in feed. The list is updated regularly and published yearly in the Association's *Official Publication*. FDA feed safety experts participate in evaluations of ingredients proposed for addition to the list. FDA recognizes the names that appear in the *Official Publication* as the common or usual name for feed ingredients, including pet food, that would be used in the ingredient listing on an animal feed product label.

25.3.2 Risks from ingredients (component B)

FDA has processes in place for limiting the recognized and serious risks that come from ingredients. But not all ingredients carry the same level of risk. Therefore, a gap identified by the AFSS Team is that FDA needs a method to distinguish among risks so it can determine which to address. Under AFSS, FDA intends to rank all identified feed hazards in relation to each other. The risk-ranking system will consider the risks from feed hazards present in incoming materials or feed ingredients, and consider the potential increase or decrease in risk from the manufacturing process. It will also consider how feed ingredients and mixed feed are handled at various stages, such as feed manufacturing, transporting, and on-farm mixing, and whether the way the ingredients and mixed feed are handled could either increase or decrease the risks.

A second gap is that FDA does not have a written process for triggering the development of official regulatory methods for detecting the presence of feed hazards. To address that gap, FDA has developed an internal standard operating procedure to ensure the availability of appropriate methods.[13]

25.3.3 Feed safety regulatory requirements (component C)

Not all aspects of feed production fall under feed safety regulatory requirements. Although the United States has regulations concerning the safety of

medicated feed (the cGMP program), the AFSS Team determined that a comprehensive program of regulatory requirements for feed safety must include safety practices for all parts of the feed chain, including manufacturing, packaging, storing, and distributing non-medicated feed ingredients and mixed feeds, so that feed hazards other than those already identified can be addressed.

In recent years, unanticipated problems have become serious issues, affecting the safety of human food and the health of animals. Those problems have included dioxin contamination in feed for food-producing animals; melamine and related compounds in pet food; mycotoxin contamination, including aflatoxins and fumonisins; and mixing errors involving selenium, vitamin D, monensin, or salt.

To address those concerns, the AFSS Team has recommended regulations designed to prevent or eliminate hazards, or reduce them to acceptable levels. These regulations will be developed through a systems approach, so that adequate control steps will be established throughout the feed ingredient and mixed feed manufacturing continuum.

The Food Safety Modernization Act (FSMA), signed into law in January 2011, is allowing FDA to establish new regulations regarding the manufacturing, processing, packing, or holding of animal food. The regulations would establish new provisions for cGMPs for the non-medicated feeds aspects of animal food and ingredient, and it would establish new provisions for risk-based preventive controls. The proposed new cGMPs are similar to what is currently in place for human food with appropriate differences and animal food facilities and apply to personnel, facilities, grounds, equipment, manufacturing, sanitation, and storage. The proposed preventive controls for animal food facilities that FDA is considering include the following:

- Having food safety plans
- Conducting hazard analyses
- Implementing controls to address hazards
- Monitoring the controls to make sure they are effective, taking corrective action when a problem is discovered
- Verifying that the corrective action was effective
- Having a recall plan in place.

Common elements of the food safety plans include hazard identification and a plan to address identified hazards, monitoring preventive controls to ensure that they are consistently applied, corrective actions to minimize the risk of recurrence of a hazard and to assess the potential for and prevent shipments of adulterated products, controls such as sanitation and security, and detailed recordkeeping. Key elements of preventive control plans for animal feed facilities include process controls, supplier controls, recall procedures, and submission of food safety plans.

The AFSS Team believes that broader regulatory requirements will result in the feed industry having greater knowledge of what it must do to ensure safe feed, resulting in fewer product recalls, greater assurances of feed safety, a reduced burden on FDA resources to respond to recalls, and increased focus on problem areas.

Also, under component C, the AFSS Team is suggesting that the cGMP regulations must be modernized to reflect the concern expressed by AAFCO and various feed manufacturing and animal production trade groups. These groups filed a citizen's petition addressing the difference between the cGMPs for licensed and non-licensed feed manufacturers and expressing their belief that the two sets of regulations should be merged into a 'single, streamlined set of regulations'. [14]

25.3.4 Reporting unsafe feed (component D)

Component D addresses the issue of reporting unsafe feed. FDA responded to that issue by implementing a requirement Congress had placed on the Agency to create a 'Reportable Food Registry'. (More information about the Reportable Food Registry is presented in Section 25.5.[15])

A second gap under component D is that not all feed manufacturers are required to submit records to FDA concerning clinical and other experiences with feed other than Category II, Type A medicated articles. FDA is in the process of developing those regulations.

25.3.5 Regulatory oversight (component E)

In the United States, regulatory oversight is done primarily through inspections and can include reviews of labeling, on-site visits, and sampling. Most on-site inspections are done by State feed control officials, using either Federal or State authority. Inspections are put under two broad categories: surveillance inspections, used to determine whether an establishment is in compliance with the regulations and its processes are under control; and compliance inspections, in which inspectors evaluate the establishment's compliance with the regulations and gather and document information about the facility's failures to comply with regulations. The inspectional observations can be used later in enforcement actions. (FDA has several enforcement actions available to it, including informal meetings; official 'Warning Letters', which tell the facility that FDA has enough evidence to take formal action if the facility does not come into compliance with the regulations; product seizures; and even criminal prosecutions of chronic violators.)

The challenge regulators in the United States face is finding ways to adequately carry out the inspection requirements for the large number of facilities involved in feed and ingredient manufacture and distribution. An approach FDA has developed and that the AFSS Team is further refining is a risk-based approach. In FY 2008 FDA implemented a risk-based

inspection program for FDA-licensed feed manufacturing facilities, and in FY 2009 FDA established a program for determining priorities for inspecting facilities for compliance with the BSE rules.

Those risk-based programs assess risk within the specific risk areas of medicated feed and BSE. For the future, the AFSS Team is looking for ways to compare risk across different programs, which will permit FDA to prioritize inspections during a given year and identify specific firms or types of establishments to be inspected so that FDA's inspection programs will have the greatest effect in reducing risks.

Although official inspections must still be carried out by FDA or other appropriate regulatory authorities (such as States), FDA is recognizing the value of third-party certification programs, under which a non-government entity conducts an inspection to determine whether a firm is correctly following the appropriate food or feed safety regulations.

In January 2009, FDA issued 'Guidance for Industry – Voluntary Third-Party Certification Programs for Foods and Feeds.' In it, FDA states, 'The [US] Federal government supports voluntary certification programs as one way to help ensure products meet US safety and security standards and to allow Federal agencies to target their resources more effectively.' The document provides an overview of the general attributes FDA believes should be part of third-party certification programs, and states that FDA may in the future provide more information about product-specific criteria that could be used for audits.

In the guidance document, FDA said that a company's participation in a third-party certification program, which would be voluntary, could benefit the company. 'For example, FDA may take into consideration an establishment's product-specific certification by a recognized certification body when determining [FDA's] establishment inspection priorities, as well as [FDA's] product entry admissibility decisions and field exam and sampling priorities.' Third-party certification may help a firm in other ways, the Guidance said, adding, 'Certification may also be useful during a foodborne illness outbreak. Establishments that are certified and have effective product tracing systems in place may be more easily and quickly investigated to be excluded by FDA.'[16]

The third-party verification concept is being extended to companies exporting to the United States. FSMA calls for the creation of the Foreign Supplier Verification Program, which requires importers to use a risk-based approach to verify that foreign suppliers produce food that is in compliance with FDA's preventive control requirements and is not adulterated. The law establishes a program that will allow third parties to certify that foreign food facilities comply with US food safety standards.

The AFSS Team also included feed 'defense' in this section, which is defense against terrorist attacks or vandalism carried out through deliberate creation of hazards in feed or feed ingredients leading to adverse animal and human health consequences.

25.3.6 Education and outreach (component F)

This component involves the training of inspection personnel to carry out new inspection programs, as well as provide education and outreach to the regulated industry and public so the regulations can be properly implemented by feed manufacturers and users.

25.4 International work to improve food safety

The Codex Alimentarius Commission has also undertaken work to address the issue of feed safety, and CVM has been directly involved. The Codex Alimentarius Commission, created in 1963, was implemented by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). It develops food standards, guidelines, and related texts under the Joint FAO/WHO Food Standards Programme. A main purpose of the Programme is to protect consumer health and fair trade in food, and to promote the coordination of work by international governmental and non-governmental organizations in the area of food standards.

CVM participated in a Codex Task Force on Animal Feeding from 2000 until 2005. The Task Force developed a Code of Practice on Good Animal Feeding, which was adopted in 2004 (and amended in 2008). Under the section about the Code's purpose and scope, it said, 'The objective of this Code is to help ensure the safety of food for human consumption through adherence to good animal feeding practice at the farm level and good manufactruring practices during the procurement, handling, storage, processing, and distribution of animal feed and feed ingredients for food-producing animals.'[17]

25.5 New emphasis in the United States on food and feed safety

The crisis in the United States set off by the adulteration of a processed wheat ingredient used in pet food and animal feed with melamine and related compounds caused the US Congress and Federal government regulators to evaluate the country's ability to ensure food and feed safety and take action when it was warranted.

The crisis highlighted the fact that imports are a significant part of a complex US food chain, and any plan to address food or feed safety must take into account the varied sources for food and feed products.

The US Congress addressed the causes of the melamine contamination crisis. Legislation developed by the US Congress was written to address concerns about hazards in pet food. However, because frequently the same ingredients that are used to produce pet food are also used to produce

animal feed, improvements to pet food safety will apply in large part to feed safety, as well.

The pet food/animal feed safety legislation was part of the FDA Amendments Act of 2007, which became law in September 2007. Many of the law's provisions apply to human medicine, but Title X of the bill contains the measures that apply to pet food and animal feed.[18]

Specifically, the legislation calls for FDA to establish rules:

- concerning ingredient standards and definitions of pet food;
- for process control standards; and
- about labeling standards (nutrition and ingredient information).

Congress directed FDA to seek comments from the public when determining how to implement the legislation. In a public notice of a public hearing called by FDA to collect public input, published in April 2008, FDA said the rules should not be limited to pet food. It said that, 'because pet food is well-integrated into the overall animal foods and feeds industry, FDA is concerned that certain new requirements, if limited to pet food only, would be impractical to implement, difficult to enforce, and ... not effectively provide the safety enhancements intended by FDAAA. Furthermore, because the standards mandated by FDAAA do not currently exist for any animal food or feed, limiting new requirements to pet food only would fail to address the broader food safety concerns associated with food intended for other animal species, particularly food-producing animals.'[19]

The legislation also requires FDA to establish an early warning surveillance and notification system to identify adulteration and outbreaks of illness caused by pet food and to notify veterinarians and stakeholders about adulteration. A system has been implemented that uses information gathered from telephone calls from consumers to FDA complaint coordinators. The system will be expanded to use an electronic portal that will be available to consumers so they can file product complaints at any time.

Additionally, the legislation called on FDA to establish a 'reportable animal food registry.' By law, food facilities, including manufacturers, processors, and packers, are required to report any food (excluding infant formula) – including animal feed and pet food – for which a reasonable certainty exists that the use of the product will cause harm or death to humans or animals. FDA launched the registry in September 2009.[20]

In addition to implementing the requirements imposed by Congress, FDA also took steps of its own in response to the melamine crisis and other import problems. In November 2007, FDA issued the Food Protection Plan (FPP), which emphasized prevention. The FPP was designed to work through a set of integrated strategies that address products' life cycles, to use a risk-based allocation of resources, and to be built on a foundation of science and modern information systems.[21]

The Administration of President Obama also made food safety a priority. In March 2009, the White House formed the Food Safety Working Group,

which was chaired by the Secretaries of the Department of Health and Human Services and the US Department of Agriculture, with several Agencies of both Departments participating. The Working Group submitted its report to the President on 1 July 2009.[22]

The Working Group's report stated that a modern food safety program must have three core principles:

1. Preventing harm to consumers is the first priority for the Working Group. The Working Group said it 'recommends that food regulators shift towards prioritizing prevention and move aggressively to implement sensible measures designed to prevent problems before they occur.'
2. The food safety inspection and enforcement efforts rely on good data and analysis, which will allow regulators to know which foods are at risk, which solutions should be used, and who should be responsible. Good data and analysis will allow the Federal government to give high priority to crucial inspections and enforcement activity, support State and local governments, and know how to evaluation outcomes of various activities.
3. To be able to identify quickly foodborne illness outbreaks and stop them, the Working Group recommended implementation of food tracing systems to shorten the time between the detection of an outbreak and resolution and recovery.

25.5.1 New food safety law

The new food safety law – FSMA – strengthens the ability of FDA to enforce rules to protect feed safety. CVM has a significant role in implementing this new legislation and helped develop rules under the new law's authority that apply to animal feeds.

The legislation is considered historic because it calls for a new, prevention-oriented food safety system, and because it includes a broad prevention mandate, with accountability aspects.

The Act also gives FDA new tools, including the following:

- Mandatory recall of products that a company fails to voluntarily recall
- Better access to records
- A more flexible standard for administratively detaining products that are potentially in violation of the law
- The ability to suspend registration of a facility if FDA determines that food from it poses a reasonable probability of serious adverse health consequences or death to humans or animals
- The establishment of a system that will enhance its ability to track and trace domestic and imported foods.

In addition, the Act has a new system of import oversight. Importers will be responsible for ensuring that their foreign suppliers have adequate

preventive controls in place. FDA can rely on third parties to certify that foreign food facilities meet US requirements.

25.6 Conclusion

In the United States, the approach to feed safety is evolving from a system characterized by specific responses to specific hazards to one that is risk-based, aimed at preventing the hazard before it becomes a human or animal health issue, and designed to be broader in scope than the previous system, so that it covers the entire feed industry.

The melamine contamination crisis resulted in the US Congress, FDA, and other Federal agencies developing a broad approach to food safety and feed safety. But even before that, CVM started the AFSS Initiative. The AFSS Team took a look at all aspects of animal feed safety and FDA's regulatory approach to it, cataloged the system by components, and identified gaps in which either the systems in place were inadequate or where no system existed. The work previously done by the AFSS Team nested easily within the approach developed by the US Congress and Federal agencies.

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26

The GMP+ Feed Safety Assurance (FSA) Scheme

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Abstract: This chapter provides a detailed description of the development and current content and characteristics of the international GMP+ Feed Safety Assurance (FSA) scheme. It also gives an overview of GMP+ International and discusses potential means of expanding the GMP+ FSA scheme into a global feed safety assurance scheme.

Key words: feed safety, safety management, HACCP, chain control.

26.1 Introduction

The GMP+ Feed Safety Assurance (GMP+ FSA) scheme is an international certification scheme, managed by GMP+ International in The Netherlands. GMP+ International started operating on 1 January 2010, when it took over the management of the certification scheme from the Dutch Product Board Animal Feed. GMP+ International is an entirely separate and independent organisation, allowing it to manage the certification scheme in an international context. In April 2012, more than 11,800 participants from more than 65 countries worldwide were participating in the GMP+ FSA scheme and this number continues to gradually increase.

Section 26.2 of this chapter gives a brief outline of the development of the GMP+ FSA scheme since 1992, along with a description of its driving factors and principles. Subsection 26.2.1 provides a summary of the often difficult lessons that have been learnt through practical experience, and the way in which these have shaped the current certification scheme. In Section 26.3, the structure and content of the GMP+ Feed Safety Assurance scheme is described, while subsection 26.3.1 is devoted to a discussion of its most important characteristics. Section 26.4 offers perspectives on participation in the GMP+ FSA scheme; the following section then provides an overview

of the additional support that GMP+ International offers to participating companies. In subsection 26.5.3 the setup of GMP+ International is discussed, along with a description of its working methods. Following Section 26.6 on feed safety culture, the chapter ends with an outline of future prospects and developments, as well as the intended targets for 2015.

26.2 Development of GMP+ feed safety policy since 1992

The GMP+ Feed Safety Assurance scheme (GMP+ FSA) started within the framework of the Dutch Product Board Animal Feed in 1992, which managed it up to the end of 2009. GMP+ International has managed this scheme since January 2010. The scheme was developed based on practical experiences and draws on lessons learnt in the field of feed safety, some of which have been extremely challenging.

In its early stages, the GMP+ FSA scheme was based solely on the principles of Good Manufacturing Practices. The primary focus was on the control of (1) cross-contamination of growth promoters (antibiotics) and coccidiostats; (2) contamination with undesirable substances; and (3) *Salmonella* contamination in compound feeds and pre-mixtures.

In 1994, the standards of the scheme were adjusted according to the ISO 9001 standard. The reason for this modification was that some companies that were used to the ISO 9001 structure wanted to transfer to the GMP+ scheme, or else wanted to combine ISO 9001 and GMP+ certification. From that point, general safety management requirements have been integrated in the GMP+ FSA scheme.

In 1999, it was decided that substantial improvements to the certification scheme were required, after several instances of serious feed contamination between 1997 and 1999, which showed that animal feed contamination could originate in the supply chain. In 1997 the Dutch agricultural industry was confronted with the first case of bovine spongiform encephalopathy (BSE). As a result, the dairy and beef industry and the cattle breeding industry experienced a dramatic reduction in the export of their products. It is important to note that the Dutch agribusiness exports about 60% of its total production, so its market position is very vulnerable in the face of feed and food safety incidents. This first case of BSE resulted in a great deal of media attention. Exports fell significantly, and the feed industry was held responsible, because meat and bone meal in animal feeds is considered to be the cause of BSE. As a result of this, and of subsequent cases of BSE, an intensive sampling programme for cattle feed was introduced. Cross-contamination made it impossible to reduce the presence of meat and bone meal traces in cattle feed. Consequently, in 1999, dairy farmers and the dairy industry began to produce and transport meat and bone meal-free cattle feeds wholly separately from other compound feeds that did contain meat and bone meal.

In 1998 Germany, The Netherlands and Belgium were confronted with a large amount of Brazilian citrus pulp contaminated with dioxin. The possible cause of this contamination was the use of certain types of waste as the fuel for direct drying of citrus pulp. Although the case did not attract a great deal of media attention, it still led to refinements being made in the practices of the feed industry. The contamination did not result in a level of dioxin in milk above the maximum permitted level; however, the dairy industry did not accept the use of the contaminated citrus pulp in dairy cow feeds. Approximately 40,000 metric tons of Brazilian citrus pulp were consequently destroyed. In 1999, another case of dioxin contamination affecting GMP+ certified companies occurred: in this instance, feed fats from Belgium were affected. The cause was co-mingling of used frying fat with some battery liquids with a very high content of dioxin and dioxin-related polychlorinated biphenols, which was then used as a feed ingredient. As with the BSE case, there was a great deal of media coverage of this contamination, heightened by political sensitivities, the stringent measures imposed, and the increasing pressure from the dairy and meat industry to enhance feed safety controls.

This accumulation of incidents of varying severity played an important role in the move to enhance the GMP+ scheme in three ways. Firstly, in 1999, it was decided that the safety of all feed ingredients and services such as transport, storage and transshipment (chain control) must be controlled in a convincing and transparent way. Between 2000 and 2003, it became obligatory for all participants in the supply chain to be either GMP+ certified or certified according to an accepted equivalent certification scheme. This measure was an important step towards increasing the international participation in the GMP+ scheme. Secondly, the scheme was made less reactive and more proactive through the incorporation of HACCP (Hazard Analysis Critical Control Points), which resulted in the name 'GMP' being changed to 'GMP+'. HACCP is an important method that allows feed safety management to be based on thorough assessment of the production process. Thirdly, in 1999 the Early Warning System (EWS) was introduced: this meant that all participants were required to inform the scheme manager of any event that occurs or may occur with regard to feed safety. Ensuring that all participants are informed of such events means that everybody will be aware of the possible risk and will be able to take preventive control measures. The monitoring and control systems put in place in the late 1990s and early 2000s were timely, as the following list of feed-safety-related incidents or crises shows:

- Salmonellae in eggs and poultry meat (1988)
- High aflatoxin in US corn gluten feed (1989)
- Residues of antibiotics and coccidiostats in eggs (1988–1992)
- BSE and meat-and-bone meal (1989, 1997, 2000)
- Dioxin in Brazilian citrus pulp (1998)

- Dioxin in Belgian feed fat (1999)
- Dioxin in German kaolin (1999)
- Nitrofen in German cereals (2002)
- MPA in Irish sugar syrup (2002)
- Dioxin in German bakery products (2003)
- Dioxin in Dutch potato by-products (2004)
- Cadmium in Chinese zinc sulphate (2005)
- Dioxin in Belgian animal fat (2006)
- Melamine in Chinese food and feed (2007–2008)
- Dioxin in German fat mixtures because of adding technical fat (2010/2011).

From 1999 on, those in the chain affected by feed contamination have increasingly held the suppliers responsible and liable for the financial losses incurred in the feed and food chain downstream. This new situation seemed to provide a useful basis for gaining more support for the improvements to the GMP+ FSA scheme that were required, and for better compliance.

One important improvement made to the certification scheme was the introduction of clear and unambiguous rules of certification in 2003. Before that year, an internal department carried out the inspections and audits and the scheme manager issued the certificates with no external involvement. The extension of the scope of the scheme and the increasing international participation forced a change in the certification policy. Several factors were important triggers for this change, including the ever increasing demands for improved audit and certification capacity, the need to separate scheme management from auditing and certification, and the requirement for risk to be reduced. Since then, the GMP+ scheme manager has emphasised the importance of clear and strict rules of certification, as this determines the liability of the certification scheme and its logo. To improve the liability, uniformity and impartiality of the certification process, certification bodies must operate under accreditation according to the norm EN 45011. The GMP+ scheme manager additionally implemented several supervisory actions in order to ensure uniform application of procedures.

26.2.1 Application of knowledge and experience acquired

Over time, the management of the GMP+ FSA scheme and the participating companies have learned a number of lessons, namely the following:

1. Proactive operation instead of reactive action by application of HACCP in a dedicated and thorough manner is necessary to reduce the chances of a feed safety incident occurring to an acceptable level.
2. A chain-focused approach is necessary in order to control feed safety from the beginning of the chain, thereby avoiding wider contamination in the following links of the chain, which would dramatically increase the financial impact.

3. The focus on the chain also means that everybody in the chain has to take responsibility for the feed safety of the products and services supplied. The 'gatekeeper' approach does not provide sufficient certainty and will not result in sufficient awareness and liability in the previous links of the chain. The 'gatekeeper' approach is acceptable and feasible in specific situations, but it should be considered a transitional measure to be gradually phased out over time.
4. The purchase of ingredients should not be impulsive and based solely on cost, but must be preceded by adequate risk assessment.
5. Beside preventive control measures, based on a profound risk assessment according to HACCP, tracking, tracing and recall plans are also necessary to limit damage in case of a contamination incident.
6. The exchange and sharing of basic information regarding hazards and risks in certain product–process combinations is crucial in order to optimise feed safety control along the whole feed chain, involving several types of companies.
7. Clear rules of certification and adequate supervision and monitoring of the certification process are essential in maintaining the credibility of the certificates and the certification scheme in the market.

26.3 Structure and content of the GMP+ Feed Safety Assurance (FSA) Scheme

The GMP+ Feed Safety Assurance scheme (Fig. 26.1) consists of four groups of documents:

A. General governance documents regarding rights and duties of certification bodies, certified companies, the use of the GMP+ FSA logo and disputes
B. A number of normative standards against which companies can become certified, covering the entire feed chain
C. A number of documents regarding the rules of certification and supervision and monitoring
D. Informative documents such as guides, research reports, etc.

The scopes of the standards in group B cover the entire feed chain from cultivation up to the delivery of feeds to the livestock farmer (Table 26.1).

The GMP+ B1 standard is an ISO 9001 structured standard, which covers most stages in the feed chain (Table 26.1). This standard can be applied by companies with an ISO 9001 quality management system or an ISO 22000 system in place, and by companies that are active in more than one stage of the chain. The GMP+ FSA scheme allows combined audits to be carried out when certification for more than one scheme or standard is required. The other standards in group B are tailor-made standards applicable for certain companies/activities in the feed chain, structured and written in a

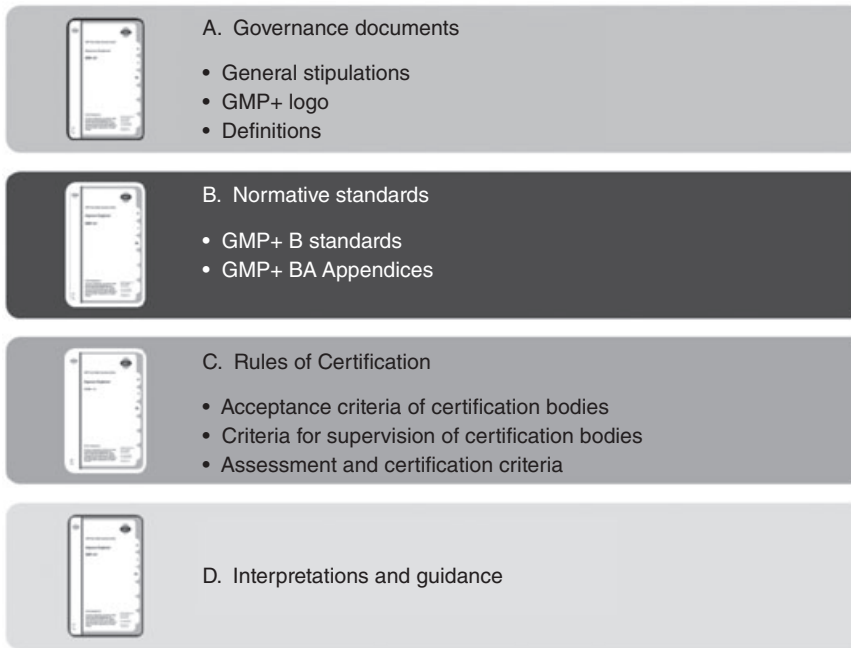

Fig. 26.1 Structure of the GMP+ FSA scheme documents.

Table 26.1 Scope of GMP+ FSA standards

Activities/products	Feed materials	Feed additives	Pre-mixtures	Compound feeds
Cultivation	×	–	–	–
Trade	×	×	×	×
Storage	×	×	×	×
Transshipment	×	×	×	×
Production/Processing	×	×	×	×
Transport	×	×	×	×
Laboratory testing	×	×	×	×

more practical way for companies which are not acquainted with the ISO approach and terminology (Table 26.2). All these GMP+ B standards are applicable worldwide by all companies active in the feed chain.

In addition to the international standards, GMP+ International allows further national specifications, for instance to comply with specific market demands related to feed safety issues or to tailor the product standards (maximum permitted levels of undesirable substances) according to different legal requirements in a certain country. The only restriction on these

Table 26.2 Situation regarding GMP+ B standards ad scope

Scopes	Standards			
	GMP+ B1	GMP+ B2	GMP+ B3	GMP+ B4
Production compound feed	×			
Production premixes	×			
Production additives	×	×		
Production feed materials	×	×		
Trade and collection	×		×	
Storage and transshipment	×		×	
Transport and chartering				×
	ISO 9001 structure	Tailor-made	Tailor-made	Tailor-made

country-specific notes is that they should not lower the level of feed safety assurance. The intention of this method of operation is to achieve the greatest possible level of international uniformity and standardisation while also allowing for national flexibility where this is necessary. This approach is essential in realising an internationally applicable certification scheme that will also receive worldwide support.

26.3.1 Characteristics of the GMP+ FSA Scheme

The GMP+ FSA scheme is a well-designed scheme, developed on the basis of substantial practical experience, including severe incidents and crises. These events were very important triggers for the development of the GMP+ FSA scheme. The most important characteristics of the GMP+ FSA scheme are listed in Fig 26.2.

Quality management requirements according to ISO 9001

The quality management requirements in the GMP+ FSA scheme are necessary to ensure consistency in the daily operations regarding feed safety management in the organisation. Quality management can be considered to have three main components: quality control, quality assurance and quality improvement. Quality management is focused not only on product quality, but also on the method by which this can be achieved. Quality management therefore relies on quality assurance and control of processes as well as products to achieve more consistent quality.

The GMP+ B1 standard in particular is structured according to ISO 9001. However, although the other tailor-made standards are not structured

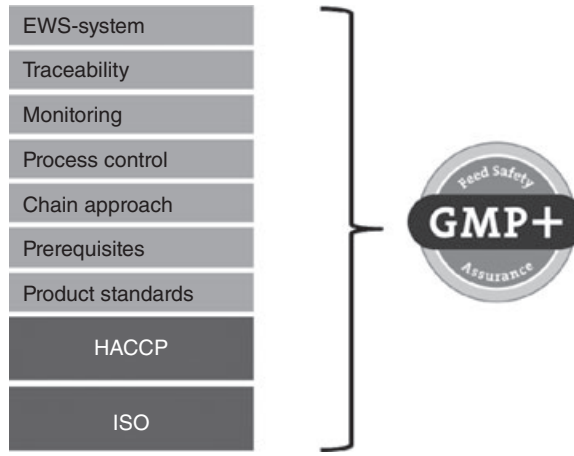

Fig. 26.2 Characteristics of the GMP+ FSA scheme.

in that specific manner, they do also contain quality management requirements comparable with ISO 9001. This onwards integration of quality management requirements facilitates the application of GMP+ FSA for ISO 9001 or ISO 22000 certified companies.

HACCP and risk assessment

In 2000, HACCP and risk assessment were integrated into the GMP+ certification scheme, transforming it from a reactive to a proactive approach to feed safety management. This concept was adopted from the food industry, which had already begun to apply it from about 1996 onwards. However, the GMP+ certification scheme took the lessons learned by the food industry into account, by involving food safety management experts in the development of a guide for implementing HACCP in the feed industry. When HACCP was first introduced into the GMP+ FSA scheme, the concrete application of the procedure needed support. Firstly, a practical guide to the application of HACCP in the feed company was introduced, developed by TNO Food (Zeist, The Netherlands) in cooperation with feed safety management specialists. Next, following a request by the industry, a number of generic risk assessments of feed materials were carried out in accordance with the method established in the guidance mentioned above. These generic risk assessments of feed materials served as a guide and a tool for the companies to carry out their own company-specific risk assessment. Thus over the course of time, all parties involved in the GMP+ feed industry learned to apply the HACCP concept correctly. This was accompanied by a change in mindset in the GMP+ family: specifically, a movement away from a low-cost-oriented approach in the short term towards a risk-reduction approach in the longer term.

A related development was the introduction in 2003 of the Database Risk Assessment of Feed Materials (DRAFM). The primary motive for the introduction of the DRAFM was the MPA (methoxyprogesterone acetate) crisis in 2002. Since that point, GMP+ participants have only been allowed to sell or buy feed materials that are listed in the DRAFM, which was integrated into the current Feed Safety Database (FSD) in September 2010. This database is not a positive list, but a list of feed materials including an available generic risk assessment. Today, this FSD (DRAFM) contains about 120 risk assessments, covering more than 500 feed materials. During the redesign of the content of the GMP+ FSA scheme in 2005, the GMP+ B1 standard was made compatible with the draft ISO 22000 that existed at that time.

Product standards

In the framework of the GMP+ FSA scheme a number of product standards are applicable. The scheme differs in this respect from generic standards such as ISO 9001 and ISO 22000. These product standards are the maximum permitted levels of undesirable substances, residues of pharmaceuticals (medicines) or microbiological agents. Most of them are legally obligatory standards (European Union) and some of them are based on specific demands in the feed and food chain. These product standards provide individual feed companies with a uniform feed safety reference for determining product specifications, which will save time and improve transparency in the market.

Prerequisite programmes on animal feed manufacturing

Since its inception, the GMP+ FSA scheme has contained prerequisite programmes on animal feed manufacturing, although to begin with HACCP as such was unknown. In the first phase, these requirements were stipulated as good manufacturing practices. Prerequisite programmes (PRPs) are all the processes and hardware that work alongside the HACCP system. They provide the basic environmental and operating conditions essential for the safe production of feeds. HACCP is product and process specific, but prerequisite programmes are more general and apply to all areas in a feed facility. Examples of prerequisite programmes include, but are not limited to, good manufacturing practices (GMPs), good hygiene practices (GHPs), standard operating procedures (SOPs) and sanitation standard operating procedures (SSOPs). The design and construction of feed premises are also part of the prerequisite programmes. These programmes create general conditions that reduce the chance of a negative influence on the safety of the feed products.

Chain approach

Based on bad experiences in the past with contamination sources in the supply chain, one of the main principles of the GMP+ FSA scheme today

is that the whole supply chain has to be covered. The chain approach means that, in principle, all suppliers of feeds (feed materials, feed additives, premixes and compound feeds) and services (transport, storage, transshipment and laboratory testing) have to be certified according to the GMP+ FSA scheme or according to another accepted equivalent certification scheme.

It is a basic principle that every company in the feed chain has to take responsibility for the feed safety of its supplied services and products. This principle is connected to a basic requirement in the feed legislation of major countries worldwide. It also matches the experience that it is more effective and efficient to control risks in the whole production chain from upstream onwards than to only have controls downstream. The chain approach means that all suppliers of feed materials and services (storage, transport, etc.) have to comply with the GMP+ requirements. An important advantage of this approach is the uniformity and standardisation of conditions and operations regarding feed safety assessment and management. It contributes not only to greater efficiency in the internal operations of companies, but especially to the efficiency of the business as a whole and to business operations, with the added benefits of saving time and improving transparency. Additionally, it prevents the distribution of contaminated lots of feed materials in the feed chain, as these will be better controlled in the upstream part of the feed chain.

The feed chain for unprocessed feed materials begins at the point of collection of grains, seeds and pulses. The collector of these products operates as the gatekeeper and executes an intake procedure including sampling, based on a risk assessment. For processed feed materials, the feed chain starts at the point when a feed material comes into existence, for instance at the moment of separation of sugar water and sugar beet pulp, or when the oil and protein content of soybeans are separated by crushing.

Furthermore, all links in the feed chain, such as trade, storage and transshipment, production of additives, of premixes and compound feeds, must be demonstrably involved in the GMP+ chain. For sea vessel transport, the requirement is that before loading, an independent load compartment inspection (LCI) must be carried out in order to determine whether the load compartments are clean and suitable for the transport of feed materials without risk of contamination.

The gatekeeper method can only be applied in specific cases in which there is a lack of demonstrable chain-wide risk control. The main reason for creating this exception is the insufficient supply of required feed materials from assured sources. That is the case with unprocessed grains, seeds and pulses of non-structural origin. If the gatekeeper method is used, all consignments have to be sampled and analysed based on parameters judged to be relevant according to a risk assessment, before they are delivered into the GMP+ chain. This kind of arrangement is also permissible for palm oil. Finally, GMP+ producers of premixes may, under strict conditions, operate as a gatekeeper for additives.

If producers of premixes and compound feed in a certain country want to become GMP+ FSA certified, but there is a lack of GMP+ certified suppliers of feed materials and transport, then during a transfer period of a couple of years a tailor-made gatekeeper solution can be arranged with GMP+ International. In that event, the interested feed companies and GMP+ International will carry out a programme to unroll the GMP+ FSA scheme in the whole supply chain of the country.

Monitoring

Monitoring requirements are also included in the GMP+ FSA scheme. This means that companies must undertake a minimum level of frequency of sampling and laboratory testing in order to verify the effectiveness of control measures and to detect possible contaminations at earlier stages. It is also possible for companies to share the results of laboratory tests, which offers savings on the cost of sampling and testing. The Feed Safety Database offers an efficient method for companies to share results. Related to the minimum sampling and laboratory testing requirement, the GMP+ FSA scheme also includes specific sampling methods and minimum quality requirements for testing laboratories.

Traceability

Traceability (tracking and tracing) requirements are stipulated so that a GMP+ certified company is able to act quickly if contamination should occur. Companies are obliged to register details of all incoming and outgoing goods. The internal processing and movement of goods must also be registered, so that the incoming and outgoing goods can be connected. In the event of a contamination, these records have two functions: (1) they enable a company to trace back (tracking) to the source and origin of contamination; and (2) they allow the company to trace forward to where the (possibly) contaminated goods have been delivered. It is then necessary to inform the customers to block and if necessary to recall these goods in order to reduce the impact of a contaminated lot for the companies downstream and to prevent further distribution of the (possibly) contaminated goods.

Early warning system (EWS)

The early warning system (EWS) means that a GMP+ certified company that notices a (possible) contamination which could also affect also participants is obliged to inform GMP+ International. In that event, GMP+ International then informs every GMP+ participant via an electronic newsletter. This is an extremely important step, ensuring that everybody is aware of the risk and able to take appropriate measures, if necessary. In this way, a potential incident or crisis can be controlled at an early stage, avoiding widespread contamination in the chain.

26.3.2 Transparency

GMP+ International strives to operate as transparently as possible. All GMP+ FSA requirements are published on the Internet (www.gmpplus.org) and publicly available. Everyone can subscribe to receive the electronic newsletters regarding changes and other relevant developments. All certified companies and accepted certification bodies are published in a public database on the Internet, including the scope and expiry date of the GMP+ certificate. If a GMP+ certificate of a company or an acceptance of a certification body is suspended or withdrawn, this will also be published in an electronic newsletter.

Although only partners (see Section 26.5.3) are involved in the decision-making process, a public consultation of drafts is one of the crucial steps in the decision-making process. This is important in ensuring the most transparent operating systems possible.

26.4 Participation in the GMP+ Feed Safety Assurance (FSA) Scheme

In 2010 about 11,600 companies were GMP+ certified, located in 65 countries worldwide (Table 26.3 and Fig 26.3). More than 50% of the participants are road transport or inland waterway transport companies, and about 40% are producers of and traders in feed materials, or involved in storage and transshipment. About 10% are producers of pre-mixtures and compound feeds.

26.4.1 Third-party certification

A process known as third-party certification, governed by uniform certification rules, is extremely important. Third-party certification is an assessment

Table 26.3 Number of GMP+ certified companies, accepted certification bodies and accepted auditors, 2006–2010

	GMP+ certified companies					Accepted certification bodies				
	2006	2007	2008	2009	2010	2006	2007	2008	2009	2010
Germany	3796	4391	4358	4635	4845	16	16	15	15	14
The Netherlands	2132	3666	3697	4309	4509	12	11	11	11	12
Rest of Europe	748	1066	1122	1402	1602	2	2	2	3	2
North and South America	359	365	348	395	439	1	1	1	0	0
Asia	184	201	231	190	214	0	1	1	1	1
Africa	14	23	15	18	9	0	0	0	0	0
Oceania	3	4	0	2	1	0	0	0	0	0
Total	7236	9716	9771	10951	11619	31	31	30	30	29

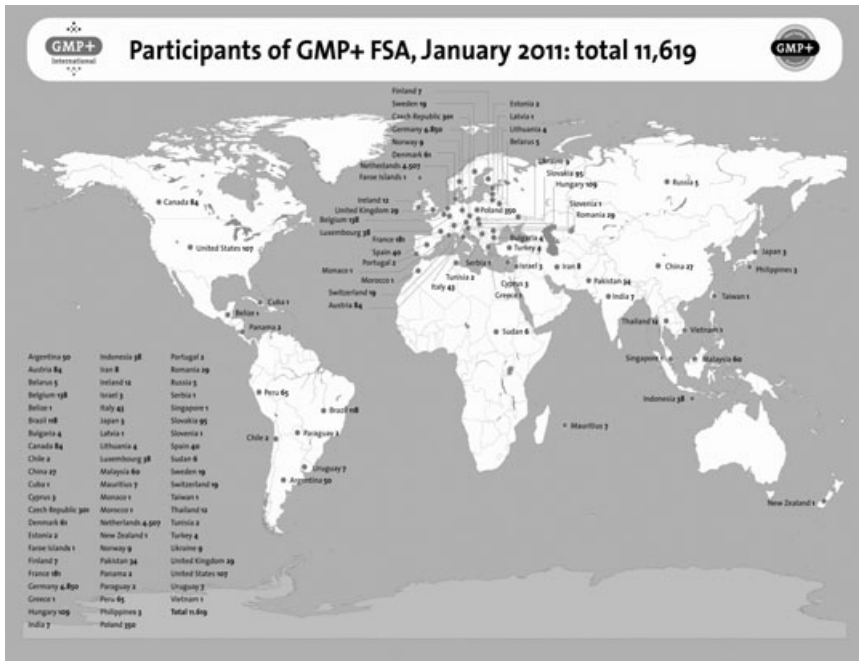

Fig. 26.3 Locations of GMP+ certified companies.

carried out to ensure that a company is complying with the requirements of the applicable GMP+ FSA standard(s). Importantly, the assessment is carried out by an independent, impartial and competent third-party organisation, which is qualified and has a licence to issue a GMP+ certificate when the assessment is successfully completed. In the C group of documents of the GMP+ FSA scheme (Section 26.3), a number of documents are published that contain the rules of certification.

First, a number of criteria must be fulfilled in order for certification bodies to become accepted and to receive a licence to issue GMP+ certificates. One of these criteria is the requirement to be accredited according to EN 45011. Accreditation is a process in which certification of competency, authority and credibility is presented. Accreditation helps to ensure that the certification practices of a certification body are acceptable and carried out in an impartial and independent way. The auditors have to comply with requirements regarding the level of professional education, level of professional skills (for the lead assessor), knowledge of the production process and products, knowledge of the applicable GMP+ FSA standard and a minimum of practical experience. Additionally, auditors have to pass periodic exams provided by GMP+ International to ensure that their competencies are ongoing.

The GMP+ FSA scheme includes assessment and certification criteria relating to the carrying out of company audits. Certification bodies must apply these assessment and certification criteria when they carry out company audits for GMP+ certification. Initial audits, supervision audits and extension audits are regularly provided.

The certification body will carry out an initial audit in order to assess whether the applicant company meets the criteria for the relevant GMP+ standard. A GMP+ certificate may or may not be granted by the certification body on the basis of this initial audit, depending on whether the determined assessment criteria have been met. The period of validity of the certificate is a maximum of three years. An initial audit is a comprehensive assessment of the quality system and consists of (1) an assessment of the quality documentation and (2) an on-site audit regarding the implementation of the requirements of the applicable GMP+ standard(s).

The certification body will carry out supervision audits, which are in principle announced, at least once a year during the period of validity of the GMP+ certificate, to assess whether the company continues to comply with the requirements for certification. The aim is that during the validity period of the certificate, compliance with all GMP+ FSA requirements is assessed at least once by means of annual supervision audits.

The GMP+ certificate may only be extended when an extension audit finds that the company still complies with all the GMP+ FSA requirements. In good time before the end of the validity period of a certificate, an extension audit must be carried out to assess compliance with all GMP+ requirements.

Alongside the audits mentioned above, some additional audits are also possible. The results of one of the previously mentioned audits may indicate that one of the following further audits is necessary: compliance audit, stricter supervision or repeat audit.

- *Compliance audit*: If one or more Category 2 nonconformities are observed, then the certification body may carry out a compliance audit. This audit is in addition to the normal audit cycle and is aimed at specific aspects related to the observed nonconformity and the improvement measures taken.
- *Stricter supervision*: In the event of the observation of one or more Category 1 nonconformities a certification body may decide to temporarily suspend the GMP+ certificate for three months. In that case, after the suspension is lifted, a certification body is allowed to place the company under stricter supervision. This will only be implemented if there are doubts regarding the attitude of the company to consistent compliance with the requirements. The stricter supervision will take place for a minimum of three months and a maximum of six months.
- *Repeat audit*: The reason for a repeat audit may be an early warning system (EWS) alert, complaints or incidents, or something else.

Depending on the nature and content of the indications, a repeat audit can be carried out by the certification body or by the certification body in the presence of a GMP+ International auditor.

To ensure that the GMP+ audits of the certification bodies at a certain level are uniform, monitoring is carried out to check that the following three requirements are met:

1. The audit must be carried out of a minimum length of time, depending on the kind and size of the company.
2. A structured checklist has to be used during the audit.
3. A number of criteria must be defined for classification of nonconformities and several measures or sanctions must be stipulated per nonconformity category.

26.4.2 Supervision of the certification process

In addition to accreditation audits by an accreditation body, GMP+ International carries out a number of supervision and monitoring activities in order to ensure uniform and ongoing applications of the rules of certification by the certification bodies. The following activities are carried out (www.gmpplus.org):

1. Technical meetings, coordination and harmonisation meetings for updating of professional expertise and standardising the interpretation of the rules in practical situations.
2. Periodic examination with respect to knowledge of the GMP+ FSA scheme.
3. Parallel audits carried out by GMP+ International at GMP+ certified companies to verify the method by which an audit is planned, executed and reported by the certification body. This parallel audit will take place as quickly as possible after the certification body's audit has been carried out and reported to GMP+ International.
4. Witness audits: GMP+ International supervises the GMP+ auditors/inspectors by assessing their working methods and the way in which they categorise their findings during the execution of their audit. The individual auditor/inspector or the audit team will be assessed during a witness audit.
5. Report assessment: GMP+ International assesses on a random sample basis the reports of audits carried out by certification bodies under the GMP+ FSA scheme.
6. Office audit: GMP+ International carries out regular audits at the certification body's office in order to verify compliance with the rules of certification, based on the results of the previous assessment activities.

26.5 Additional support of companies

Besides the management of the GMP+ FSA scheme, GMP+ International offers several additional services to GMP+ certified companies, namely:

- GMP+ Feed Safety Database (FSD)
- Training and education regarding feed safety by the GMP+ Academy
- Literature studies, guides, etc.

26.5.1 Feed Safety Database

The Feed Safety Database (FSD) is a web-based portal that offers access to a number of interesting information sources, such as generic risk assessments of feed materials, test results of undesirable substances and microbiological agents, fact sheets regarding contaminants and product standards (maximum permitted levels).

The FSD contains about 200 generic risk assessments covering around 500 feed materials. These generic risk assessments are a useful resource for companies to carry out their own, company-specific risk assessment: they will contribute to a harmonised approach to company-specific risk assessments and will be a guide for the auditors of the certification bodies. These generic risk assessments also contribute to the development of a uniform and standardised method of carrying out risk assessments in the whole feed chain.

The FSD contains the test results of around 350,000 samples, analysed from about 1980 onwards. It is a useful source of information for studies on general trends in food safety, but can also be helpful in risk assessments.

The fact sheets provide a great deal of relevant information the undesirable substances and microbiological organisms for which a maximum permitted level in feeds has been established: this will assist companies to carry out their own risk assessments, for instance to determine the severity of hazards. The content of every fact sheet is as follows:

General summary
Summary of GMP+ product norms for the animal feed sector
More facts
1. Nature, history and prevalence
2. Transmission to the environment, plants, animals and humans
3. Diagnosis of poisoning
4. Potential hazards and adverse effects
5. Severity of the hazard
6. Standards
7. Analysis methods
8. Control measures
9. References
10. Websites
Appendix I Toxicity data

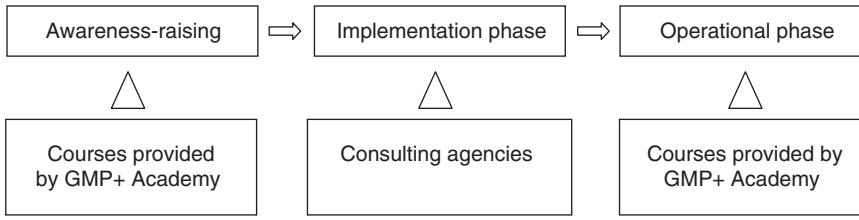

Fig. 26.4 Activity fields of the GMP+ Academy.

These fact sheets explain the severity of the hazard. Reference to these fact sheets allows GMP+ certified companies to base their risk assessment on a uniform and common (scientific) base of knowledge, which in turn contributes to best feed safety control practices, and means that companies do not have to spend time collecting this scientific information.

26.5.2 GMP+ Academy

Since January 2011, GMP+ International has offered training courses regarding feed safety management and feed safety related issues under the name 'GMP+ Academy'. The training courses are developed and carried out by the members of the GMP+ Academy, and aim to transfer knowledge about feed safety management to all levels of feed companies and auditors of certification bodies in order to achieve maximum safety assurance in the feed chain. The GMP+ Academy aims to offer training courses that match both the awareness-raising phase and the operational phase (see Fig. 26.4).

The training courses are mainly led by the members of the GMP+ Academy. Members are training institutes or scientific institutes with a good international and/or national reputation. Until mid-2011 the GMP+ Academy will be made up only of Dutch and German institutes, but the intention is to extend the number of GMP+ Academy members in order to be able to offer training courses in all relevant countries in their own language.

26.5.3 GMP+ International

From 1992 until 2009 the GMP+ Feed Safety Assurance scheme was managed by the Dutch Product Board Animal Feed, a semi-governmental organisation. In 2009, it was decided to transfer the management of the GMP+ FSA scheme to a private, internationally operating non-profit organisation. The main reasons for the privatisation of the management of the GMP+ FSA scheme and its related activities are as follows:

- The increasing number of participants internationally (at the beginning of 2009 there were more than 10,000 participants in 65 countries worldwide).

- Increasing interest from stakeholders outside The Netherlands, who wanted to be involved in the decision-making process.
- Increasing interest in the application of the GMP+ FSA scheme for the delivery of feed materials and premixes to destinations outside North-west Europe.
- The embedding of the scheme in a national framework proved to be an obstacle to its transformation into a genuinely international scheme, accepted in an ever increasing number of countries.

In January 2010 GMP+ International started as a new legal entity, and further elaboration of the new organisation was undertaken. The structure and method of operating is now focused on the following aspects:

- Management of an international, chain-oriented feed safety assurance scheme applicable worldwide.
- Allowing for specific national needs via additional country notes.
- Support, involvement and engagement of stakeholders in the feed and food chain via international partnerships.
- Interdependency, thanks to well-balanced participation of all stakeholders in the expert committees.
- A non-profit making approach.

In the vision of GMP+ International, the feed chain is part of the animal production chain. That whole chain operates to an increasing extent on an international level. Ultimately what is important is ensuring the safety of the animal products that are offered to theconsumer at the end of the chain. GMP+ International works on the principle that every company is itself responsible for the correct assurance of the safety of the products and services which it supplies. GMP+ International also holds the opinion that the various links in the feed chain have a shared responsibility for the determination of minimum prerequisites for the assurance of feed safety in the whole feed chain. This is because of the interdependency in the system ('the chain is as strong as the weakest link') and the need for uniformity and standardisation. The core values of the organisation are reliability, independence, a chain-oriented approach and high-level performance.

The mission statement of GMP+ International is to actively coordinate and promote a uniform and standardised application of basic principles for the assurance of feed safety in the whole feed chain worldwide and to contribute to the production of safe food of animal origin. GMP+ International continuously responds to the wishes of its participants, and works in cooperation with the stakeholders in the feed and food chain, following the latest scientific insights and practical experiences, with due observance of the core values mentioned above.

GMP+ International also seeks the support, involvement and engagement of interested trade associations in the feed and food chain that may wish to become partners of GMP+ International. For GMP+ International

it is important to have partners worldwide that share and support the vision, values and ambitions of GMP+ International. All trade associations that represent companies in the feed and food chain are allowed to become partners, on the condition that they endorse the Code of Principles. This code contains principles expressing the vision, values and ambitions of GMP+ International regarding its certification scheme for feed safety assurance.

The Code of Principles contains, for instance, the following principles (www.gmpplus.org):

1. GMP+ International's partners are committed to responding to the concerns of the animal production chain and to consumer concerns relating to food safety in an appropriate, adequate and convincing manner. Both human health and animal health are included in this approach, and human and animal health surpass economic and commercial interests.
2. GMP+ International's partners encourage continuous improvement, development and understanding of the best practices in feed safety assurance in a trustworthy, conscientious and consistent manner. Best practices are related to the latest insights regarding (1) quality assurance management, (2) Hazard Analysis Critical Control Points, (3) the application of good manufacturing practices (prerequisite programmes) and (4) traceability.
3. GMP+ International's partners endorse that preventive and corrective measures are based on the latest scientific knowledge, but in case of doubt or lack of knowledge, the precautionary principle will have to be applied.
4. GMP+ International's partners endorse the establishment and maintenance of an international, chain-oriented certification scheme which serves as a basis for all types of feed companies (maximum uniformity with respect to the essential aspects of feed safety assurance), recognising the mutual dependence of all links in the feed chain. This also involves aiming for specific, national (end) product standards to comply with additional national requirements.
5. GMP+ International's partners recognise and endorse the shared responsibility of every company and link in the feed chain for the common interest of an optimal feed safety assurance in the entire feed chain, in order to comply with legal requirements and feed safety assurance related to downstream (animal production) food chain demands.
6. GMP+ International's partners endorse also the application of clear rules for third-party certification under accreditation and for supervision of the certification practices in order to achieve uniform, reliable and solid certification and a high GMP+ compliance performance within the participating companies.

The main benefits to partners are the following:

- To be allowed to nominate candidate members for the International Expert Committee and for international and national subcommittees and technical committees for the scope of economic activity they represent
- Access to all draft documents that are being prepared for the technical and subcommittees and the International Expert Committee and their proposals
- Access to all early warning messages
- Access to all GMP+ International's publications
- Access to the Feed Safety Database.

As of the end of 2010, GMP+ International had 19 partners, all of which are trade associations representing the different subsectors in the feed chain, including livestock farming and the processing industry. The aim is for this number to be dramatically increased in the near future (see Section 26.7). The most important parts of the organisational structure are shown in Fig. 26.5.

The International Expert Committee has the task of providing GMP+ International's management with advice (even if this has not been requested), with respect to the certification scheme and the supervision of the implementation of certification. The International Expert Committee advises on the following matters (www.gmpplus.org):

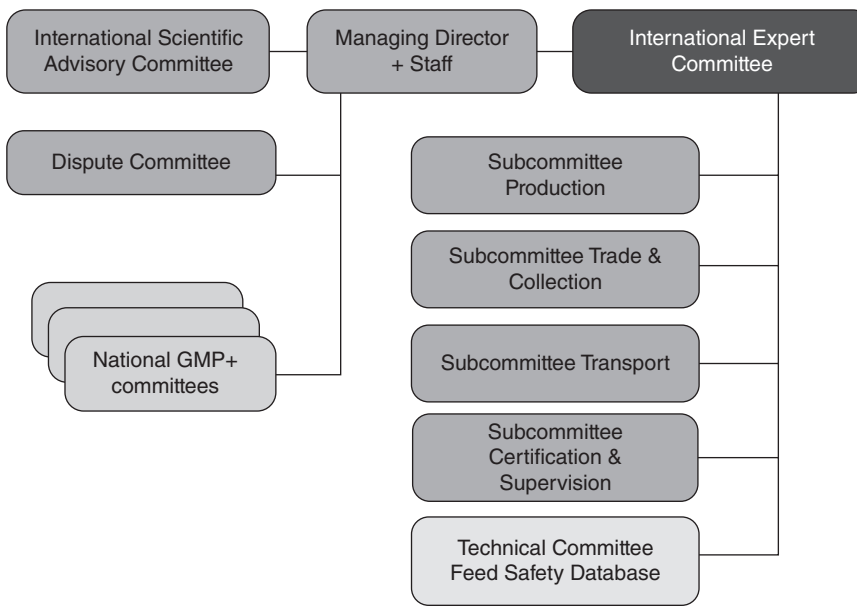

Fig. 26.5 Organisational structure of GMP+ International.

- The structure and content of the international standards which belong to the certification scheme
- The certification requirements used by certification bodies during certification
- The requirements for the approval of certification bodies and the method of working and the assessment criteria during the implementation of the supervision of the functioning of the certification bodies
- The congruency of additional GMP+ country notes with the international standards as specified in the first item in this list
- The substantive (with respect to criteria and procedures for the assessment) equivalency of other certification schemes to the certification scheme, with respect to the standards, the certification requirements and also the supervision of the certification with a view to arriving at mutual recognition or acceptance
- The use of the GMP+ logo.

The International Expert Committee establishes proposals and amendments with respect to the matters specified above and will provide advice to the management of GMP+ International, which can subsequently decide between the following options:

- acceptance of the advice in its entirety, or
- modification of the proposal with a request for the International Expert Committee to reconsider the advice, or
- rejection of the advice in its entirety.

If the management of GMP+ International modifies or rejects the advice as specified above, then the International Expert Committee will provide updated advice.

The International Expert Committee has an independent chairperson and a maximum of 17 members from the subsectors of the feed chain as specified in Table 26.4. The International Expert Committee aims to achieve consensus on the advice to be given. A decision will be taken based on a simple majority of the votes in a meeting at which at least half of the number of members or their deputies are present. In the event of a tied vote, the proposal will be rejected.

Some permanent subcommittees have been established to prepare proposals for specific subsectors or subjects. These subcommittees allow broader involvement and participation of stakeholders in the decision-making process and contribute to the operation of the International Expert Committee. Only representatives of the partners participate in the international subcommittees and the International Expert Committee. Nevertheless, all the other stakeholders who are not involved are also able to provide comments during the public consultation phase of the decision-making process (Fig. 26.6).

Table 26.4 Composition of the International Expert Committee of GMP+ International

Subsector	Number of committee members
Production of dry feed materials	1
Production of moist feed materials	1
Production of feed additives	1
Trade in and collection of feed materials Storage and transshipment of feed materials and feeds Treatment of feed materials	3
Forage trade	1
Transport (inland waterway shipping and road transport)	2
Production of pet foods	1
Production of premixes and compound feeds	4
Livestock farming	1
Processing, wholesale and retail of animal products for human consumption (food sector)	2
Total	17

Fig. 26.6 Decision-making process regarding content of the GMP+ FSA scheme.

Within the framework of GMP+ International, it is possible to establish national GMP+ committees when this is needed in a country. This allows stakeholders to be involved at the national level. The task of a national GMP+ committee can differ according to the requirements of the country and the current situation. The following are the most important tasks and responsibilities of a national GMP+ committee:

1. Translation of the (relevant) documents of the GMP+ FSA scheme into a national or regional language

2. Establishment of an additional, GMP+ country note to comply with the national or regional market demands or differing national legislation regarding feed safety
3. Promotion of the GMP+ FSA scheme to the national feed industry of the country to encourage their participation in the GMP+ FSA scheme, as well as to the animal production industry (livestock farming and meat, egg and dairy industries), retail if relevant and applicable, and the relevant governmental institutes and authorities
4. Advising GMP+ International of drafts of (modification of) international GMP+ standards, supporting activities and any other feed safety related issues
5. Advising GMP+ International of the unambiguous application and interpretation of the GMP+ standards in relation to the national or regional situation in the feed sector
6. Creation of a consulting or advisory platform or a helpdesk for (potential) participants, in order to comply with the GMP+ FSA scheme.

The GMP+ Disputes Committee is tasked with adjudicating in all disputes which may arise between participants and certification bodies as well between participants and GMP+ International. The Dispute Committee consist of members who have no direct connection with certification bodies (www.gmpplus.org).

26.6 Feed safety culture

The dioxin scandal in northern Germany, which started in December 2010, was the result of an incorrect mindset. It showed that the lack of a feed safety culture in the feed chain poses risks that also affect the food business. Good tools for feed safety will not be sufficient to address this issue.

Excellent tools are available, such as the GMP+ FSA scheme, the supervision of certification, the training programmes and a Feed Safety Database. By using these tools consistently and correctly, the risk of contamination can be significantly reduced. However, the correct use of the tools is dependent on the company mindset.

Two important factors determine this mindset. First of all, it is important that the management of a company concludes on the basis of a *rational consideration* that it is in the company's *own* interest to guarantee the safety of feed. It is a matter of choosing between the continuity of the company in the longer term and high profits in the short term. Experience has taught us that fraudulent actions or intended misconduct will eventually be exposed. It is also a matter of choosing between profitability and the liability for damage claims.

A second important aspect that determines the mindset is the *culture* in the company. The management/entrepreneur plays a leading role in this

respect. Culture is about values and standards, and about what the company stands for. It consists of responsibility for one's employees, one's customers and one's colleagues. It is also a matter of being accountable to third parties with the conviction and awareness that a company has a social responsibility and is part of the entire animal production chain. Finally, it is a matter of credibility: it must be possible for customers to have confidence that a company will keep its promises.

26.7 Future trends

The international support and involvement of stakeholders via partnerships is very important, so GMP+ International will look to substantially increase its number of partners with the aim of having 50 partners worldwide in 2015. This goal shows the intention of GMP+ to achieve international stakeholder support and involvement. GMP+ International also continues to improve the quality of its services, and will look to extend its services if there is a need in the feed business.

In 2011 the scope of the GMP+ Feed Safety Assurance scheme will be extended to (other) sustainability aspects that are relevant to the feed chain. The animal feed sector receives an increasing number of requests for sustainable operations of various types from the market and the authorities. This includes, for example, the use of soya beans, palm oil and fish meal whose production methods do not harm humans, animals or the environment. In order to demonstrate sustainable production, the sector would have to work with various certification systems. However, companies would prefer multiple aspects to be embedded within a single certification scheme. This prevents regulatory overlap, ensures uniform standards and conditions and allows the administrative burden of certifications to be limited. A single audit would allow multiple factors to be inspected at once.

The aim is to improve and extend the functionalities of the Feed Safety Database in order to increase its value for participating feed companies. This involves more efficient data entry through standardisation of the digital communication tools, and enables the storage and retrieval of test results and risk assessments. The intention is to allow companies to have their own 'room' (MyFSD) in the FSD where they can store information and communicate this information to their customers.

The ultimate intention is the establishment of an International Scientific Advisory Committee (ISAC). One of GMP+ International's aims is to operate at the highest level and to apply the newest scientific insights in its services. In order to achieve this, GMP+ International will develop a research programme to be defined by and carried out in close cooperation with the intended ISAC.

GMP+ International is preparing a complete revision of its integrity policy (rules of certification and supervision programme) in order to ensure

that it is optimised. The possibility of using forensic assessment methods to track down intended malpractice will be investigated. The minimum obligatory training and knowledge requirements for auditors, as well as the periodic assessment of these auditors, will also be improved.

To improve communication between participants and to create the possibility of international feedback, GMP+ International intends to develop a GMP+ Community, by using suitable Internet facilities and social media. This tool could be helpful in improving involvement and engagement, and in the sharing and exchange of information and experiences.

26.8 Acknowledgements

I would like to express my gratitude to the members of the motivated and dedicated team of GMP+ International. Also all the support from company experts, trade associations, consultancies and scientific research institutes past and present was and remains very important for the development of the services offered by GMP+ International.

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