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An overview of tests for animal tissues in feeds applied in response to public health concerns regarding bovine spongiform encephalopathy

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Summary

Enforcing the ban on meat-and-bone meal in feed for farmed animals, and especially ruminants, is considered an important measure to prevent the spread of bovine spongiform encephalopathy. The authors describe current analytical methods for the detection and identification of animal tissues in feed. In addition, recently approved requirements, such as the ban of intra-species recycling (practice of feeding an animal species with proteins derived from the bodies, or parts of bodies, of the same species) are described. In principle, four different approaches are currently applied, i.e. microscopic analysis, polymerase chain reaction, immunoassay analysis and near infrared spectroscopy or microscopy. The principal performance characteristics of these methods are presented and compared, and their specific advantages and disadvantages described. Special emphasis is also placed on the impact of rendering conditions, particularly high temperatures and on the use of molecular biology techniques.

Keywords

Animal feed – Bovine spongiform encephalopathy – Immunoassay – Legislation – Meat-and-bone meal – Microscopy – Near infrared spectroscopy – Polymerase chain reaction – Species identification.

Introduction

The earliest case of bovine spongiform encephalopathy (BSE) was confirmed in the United Kingdom (UK) in 1987 (4). Legislation designed since then to eradicate BSE has been based on scientific opinions, regarding both the possible responsible agent and the probable pathways of transmission of the disease. The theory of feed-borne contamination through infected ruminant protein being the major vehicle for the transmission of BSE in cattle has been widely supported by epidemiological studies, rendering studies and ultimately, by the effect of the feed bans (4, 49). Ruminant proteins enter the feed chain mainly in the form of meat-and-bone meal (MBM). Meat-and-bone meal is defined as processed mammalian tissue (exclusive of added blood, hair, hoof, horn, hide trimmings, manure, stomach and rumen contents, except in such amounts as may occur unavoidably in good processing practices) intended for animal consumption, treated so as to be suitable for direct use as feedstuff or as an ingredient in feedstuff for animals. Meat meal is also frequently included under this acronym.

Meat-and-bone meal was often introduced in concentrates to supply essential amino acids to lactating and fast-growing animals. However, MBM may also enter the diets of animals accidentally or may be present in feed as a result of cross-contamination at feed mills, during transportation, storage or at the farm.

Since the consumption of infected material by ruminants has been unquestionably recognised as the main BSE transmission pathway, legislation has been established throughout the world to avoid MBM entering the ruminant feed chain. Proteins derived from mammalians were prohibited in ruminant feed in the European Union (EU) in 1994 (24) and in the United States of America (USA) in 1997 (74). The World Health Organization (WHO) has recommended that all countries should ban the use of ruminant tissue in ruminant feed since 1996 (82). Furthermore, since the end of 2000, the EU has extended this prohibition to processed proteins derived from mammals, birds and fish for all farmed animals which are kept, fattened or bred for the production of food (31).

Enforcing legislation throughout the world requires the appropriate analytical tools. The aim of this paper is to describe the current, state-of-the-art analytical methods used for the detection of animal tissue in feed. A background on EU legislation regarding the use of processed animal by-products is also provided with an explanation of the required performance criteria for the various analytical methods.

Legislation in the European Union and methods for detecting animal tissues in feedstuffs

Ban on mammalian meat-and-bone meal in feeds for ruminants

The BSE epidemic originated in the UK, causing more than 182,000 cases between 1988 and 2002 (64). The number of BSE cases in the UK accounts for about 98% of the cases acknowledged throughout the world during that period, with about 3,100 cases being reported in the rest of the EU and 450 cases in the rest of the world. Unsurprisingly, the EU has been at the forefront in trying to reduce the risk associated with BSE and to regulate the utilisation of MBM in animal nutrition through legislation.

The earliest ban on ruminant feedstuffs containing protein derived from mammalian tissues was introduced at the EU level in 1994 (24). These measures play a critical role in the BSE eradication programme, together with legislation established in the same year on rendering systems for processing ruminant waste (25) and legislation passed in 1996 on the application of pressure-cooking systems (26). In addition, those tissues presenting a specific risk of transmitting BSE to ruminants, referred to as specified risk material (SRM) (27, 30, 33, 35), have been ascribed particular management. Finally, additional legislation has been designed for specific ruminant-derived materials other than MBM, such as hydrolysed protein and fat, regulating processing methods for their manufacture (29, 34).

The MBM ban imposed in 1994, proposed a derogation whereby, if European member states could enforce a system allowing the distinction between animal proteins of ruminant origin from animal proteins of non-ruminant origin, the feeding of protein from species other than ruminants to ruminants was permitted (24). However, since none of the member states had the tools to prove the origin of the protein, the ban applied to MBM obtained from all mammals.

The lack of adequate control methods, including a sensitive and reliable technique to discriminate and quantify ruminant protein, led to various amendments of the 1994 legislation. A decision was finally made in 2000 that processed animal proteins should be banned for all farmed animals kept, fattened or bred for the production of food (31, 71).

This total ban mainly reflects the fact that strict implementation of important measures against BSE could not be assured. The Scientific Steering Committee (SSC) of the European Commission expressed various opinions on the risk associated with the transmission of BSE through recycling of animal by-products in feed for farmed animals other than ruminants, and none of them actually recommended banning MBM provided that four crucial conditions were fulfilled, as follows (71):

– the raw material is fit for human consumption

– specified risk material (SRM) and fallen stock are removed

– pressure-cooking standards are respected (133°C, 20 min., 3 bar)

– control of the MBM ban to ruminants is effectively implemented.

If these conditions could be respected and cross-contamination avoided or detected by means of specific analysis, the ban of MBM could have been confined only to ruminant feed (71).

An overview of European legislation regarding the use of animal by-products is provided in Table I.

The problem of cross-contamination

In the 'early years' of the BSE crisis, no evidence regarding the size of the minimum infective dose or the extent of any cross-contamination in feed mills was available. In 1998, BSE cases throughout Europe were recognised as increasing at a rate higher than expected, particularly considering that the EU-

Table I
Principal European Community (EC) legislation on bovine spongiform encephalopathy (BSE) regarding animal feed

Year	Legal text and contents
1989	D 89/469/EEC Restrictions on the despatch of certain live cattle from the United Kingdom
1994	D 94/381/EC Ban on the use of proteins derived from mammalian tissues for feeding ruminants
	D 94/382/EC Rendering systems for processing ruminant waste into meat-and-bone meal (MBM) (inactivation of bovine spongiform encephalopathy agents)
1995	D 95/29/EC Amendment of D 94/382/EC – Batch rendering systems
	D 95/60/EC Amendment of D 94/381/EC – Derogation to the feed ban
1996	D 96/449/EC Pressure-cooking system for processing mammalian waste into MBM (inactivation of transmissible spongiform encephalopathy agents)
	D 90/667/EC Disposal, processing and trading of animal waste
1997	D 97/534/EC Prohibition of the use of specified risk material (SRM) (mainly brain, eyes and spinal cord)
	D 97/735/EC Restrictions on trade in MBM
1999	D 1999/534/EC Conditions for the production of MBM and tallow (repeals D 96/449/EC)
2000	D 2000/418/EC Prohibition of the use of SRM (repeals D 97/534/EC)
	D 2000/766/EC Temporary ban on use of MBM
2001	D 2001/2/EC Amendment of D 2000/418/EC – Extension of the list of SRM (bovine intestines)
	D 2001/9/EC Conditions for feeding certain animal proteins
	D 2001/25/EC Prohibition of the use of dead animals in the production of animal feed
	D 2001/165/EC Amendment of D 2001/9/EC – Hydrolysed proteins
	D 2001/233/EC Amendment of D 2000/418/EC – Extension of the list of SRM (vertebral column)
2002	R 270/2002 Amendments of R 999/2001 – SRM, surveillance, animal feeding and placing on the market of ovine and caprine animals and products thereof
	R 2002/248/EC Amending Council D 2000/766/EC and Commission D 2001/9/EC with regard to the feeding of animal proteins
	R 2002/1774/EC Laying down health rules concerning animal by-products not intended for human consumption

D : Decision
R : Regulation

wide feed ban had been in place for four years and in some countries (i.e. France, the UK), for even a few additional years. According to farmers, infected cows were never knowingly fed diets containing mammalian or ruminant protein. Unintentional cross-contamination of ruminant diets with feed intended only for monogastric and poultry species was therefore concluded to be the primary cause of the continuation of the BSE epidemic after the 1994 feed ban was implemented. Indeed, in the EU (apart from earlier national restrictions in the UK, Sweden and Portugal), mammalian MBM, therefore including ruminant protein, continued to be allowed for use in feeds for non-ruminants for about six years after the 1994 ban (24).

Cross-contamination at feed mills is probably the principal cause of ruminant feed contamination with ruminant protein: milling plants are not self-cleaning and raw materials adhere to surfaces and conveyers. They build up agglomerations that are carried over into subsequent formulations. A human or software error in the formulation of rations may also occur, together with logistic mistakes (storage in the wrong containers, mistaken trucks, errors made by the importer at store level).

In principle, the 1994 ban (24) was sound and would have been effective in eradicating BSE if most of the feed production systems in Europe had not been of mixed-species type, with 90% of the mills sharing the same facilities for the production of both ruminant and non-ruminant feedstuffs.

According to inspections carried out by the European Commission (EC) Food Veterinary Office (FVO) from 1998 to 2000 (32), a significant risk of cross-contamination of ruminant feed arose in most member states from the following:

a) failure to adequately monitor the feed ban, as follows:

– lack of a validated species-specific method to test for the presence of ruminant MBM

– insufficient sensitivity of the method used, although the limit of detection (LOD) of feed microscopy can reach 0.01% MBM in feed

– inadequate number of samples analysed

b) inadequate implementation of measures by member states

c) lack of rigour and consistency in implementation within and between member states

d) the non-establishment of dedicated feed mills in all member states for separate processing of feed for ruminants and non-ruminants.

In 1998, the SSC adopted the opinion that mammalian MBM may be a cross-contaminant of all animal feedstuffs (69).

Only two years later, the risk of cross-contamination was fully acknowledged by the SSC. This ultimately led to the prohibition of the use of any feed containing MBM (70). Only a 'zero level' of cross-contamination of feed could exclude any associated risk and adopting the necessary measures during production, transport, storage and processing of all raw materials and feedstuff produced was therefore crucial. The opinion matured after recognition that cross-contamination was practically unavoidable in the absence of species-dedicated feed mills in most plants in Europe, together with the lack of a validated test capable of differentiating between protein from different species (particularly ruminants versus other species). Legislation banning processed animal protein for all farmed animals (except for a few products for some animals, as indicated in the legislation) finally came into effect in the EU on 1 January 2001 (31). In the UK, a total MBM ban had already been implemented five years before the EU-wide total ban (2).

A definitive European regulation laying down health rules concerning animal by-products not intended for human consumption was approved on 3 October 2002 (36). The regulation controls the exclusion of dead animals ('fallen' livestock) and other condemned materials from the feed chain by introducing new restrictions, and consolidates and simplifies previously scattered legislation in one document. The key feature of this legislation is that only materials derived from animals declared fit for human consumption following veterinary inspection may be used for the production of feeds. In addition, for the first time, the regulation introduced the ban of intra-species recycling. In this respect, the availability of an effective species-specific test to identify constituents in animal feedstuffs is certainly crucial.

Tests for the identification of animal protein in feed

Currently, the only official method available for the detection of mammalian MBM in animal feed is inspection by feed microscopy. This technique was described in European legislation in 1998 (28). The method depends largely on the presence of bones and allows differentiation between bones of terrestrial animals and those of fish, although the detection of muscle tissue indicates general contamination with animal material. In addition, depending on the experience of the microscopist, and in ideal conditions, the method can distinguish between and quantify mammalian bones and poultry bones. However, the method is time-consuming, laborious, requires skill and incurs considerable cost and delay. Furthermore, the results depend on the experience of the microscopist. A detailed description of feed microscopy is given below.

Rapid control methods, capable of high throughput of samples, allowing for species differentiation and providing reliable identification are urgently required to enforce legislation on the use of MBM in animal nutrition (36). In addition, the tests also need to comply with the imposed thermal processing standards. The total ban on MBM, which will be lifted in Spring 2003, was adopted in reaction to the unfeasibility of enforcing the previous legislation (24) due to the lack of definitive and reliable methods capable of detecting unintentional cross-contamination.

In 1998, a Committee of Experts of Methods Analysis (CEMA) of the European Directorate General of Agriculture was asked to discuss, in a workshop, state-of-the-art candidate methods for identification of animal ingredients in compound feeds (53). The advantages and disadvantages of classical microscopy, enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and near infrared spectroscopy (NIRS) were evaluated (Table II). These techniques (with the exception of ELISA) are now being evaluated in an EC project (STRATFEED), that was initiated in January 2001 (3).

Currently, identifying the tools that will permit enforcement of the bans implemented to eradicate BSE is vital to comply with recent animal by-product regulation (36). The low LOD required, the complex matrices investigated and the processing conditions applied to raw materials and imposed by the legislation are technically challenging. Nevertheless, in addition to improving the existing reference methods, some promising new techniques are being investigated that show encouraging applications.

Microscopic detection of animal by-products in feeds

The EU legislation defines basic guidelines for the identification of constituents of animal origin in animal feedstuffs by microscopic analysis (28). Fine structures originating either from mammals, poultry or fish are visible on microscopic inspection at various magnifications. Chemicals and details of the procedure may be replaced freely with alternatives and additional procedural aspects can be used when necessary, provided that the results are comparable. However, inter-laboratory harmonisation of slide preparation and examination is compulsory for comparable results to be obtained. Legislation is an important tool to reach that objective and up to now, microscopic examination of feeds has been the only method validated successfully by collaborative tests (23). Microscopic detection of animal products in feeds is discussed below and future developments indicated.

Procedure

A feed specimen (minimum 100 g) is ground to pass a 2 mm exit sieve. This yields particles that are much smaller than 2 mm. After sieving 5 g of the ground sample, usually with a mesh size of 250 µm, two fractions are obtained (Fig. 1).

If the feed is contaminated with MBM, both fractions may contain bone particles, muscle fibres, hairs or feather filaments and egg shells or fish scales besides the usual vegetal feed ingredients. Simultaneously, 5 g of the ground sample is used for sedimentation in a separation funnel with tetrachloroethylene (Cl2C = CCl2). Most of the 5 g sample floats because of the relative density of tetrachloroethylene (d = 1.62). Only the more dense matter, i.e. mainly bone particles and minerals, will form the sediment. After drying, the sediment can be sieved through a mesh size of 250 µm, yielding two additional fractions. The fractions are examined as indicated in Figure 1.

The advantage of the sedimentation procedure is the concentration and selection of bone particles from the feed. A modification of the sedimentation procedure is the so-called 'French method', which uses two different solvents. This results

Table II
Currently available tests for the detection of animal protein in animal feedstuffs

Method	Analytical features	Weaknesses	Future developments
Microscopic analysis	Identifies: – animal constituents in feedstuffs – fish versus terrestrial – mammalian versus non-mammalian Limit of detection (LOD) up to 0.01% meat-and-bone meal (MBM) in feeds Test is heat-stable	Essentially based on the presence of bone fragments Not applicable on liquid samples Costly (instruments and work time) Requires experienced analysts Use of organic solvents for sedimentation of bone fragments	Only European Union recognised official method (28)
Polymerase chain reaction (PCR)	Identifies taxon and/or species-specific deoxyribonucleic acid (DNA) as marker of animal origin (i.e. vertebrates, mammalian, ruminant, bovine, sheep, goat, pig, poultry, horse DNA) LOD up to 0.1% (less if performed on sediment, probably up to 0.01%) Forensic validity Does not require the presence of bone fragments Applicable on liquid samples	Costly (equipment and reagents) Requires confined and dedicated facilities to avoid false-positive results due to cross-contamination Opportunity to test the stability of target amplicons in properly treated MBMs Heat-sensitive but at high temperatures (>140°C) Interference with DNA present in some still allowed animal proteins in ruminant nutrition (i.e. milk) or other animal products (fat, blood,etc.). In such cases, a sedimentation as clean-up step is required (see microscopic method)	Most promising for species-specific method Encouraging developments towards test heat-stability
Enzyme-linked immunosorbent assay (ELISA)	Identifies species-specific antigens Rapid, low costs (possible use as a screening test) LOD up to 0.01% Direct detection of proteins, does not require bones Applicable on liquid samples	Possible interference of ruminant products (i.e. milk) and possible inhibition by gelatine Heat-sensitive at high temperature Cross-reactivity with plant proteins	Encouraging developments towards test heat-stability Further development in compound feeds required
Near infrared microscopy (NIRM)	Identifies: – animal constituents in feedstuffs – fish versus terrestrial Does not require the presence of bone fragments Detection is performed on all the particles (i.e. muscle, bones) in the animal feed No interference of milk Quantitative analysis Test is heat-stable LOD up to 0.1%	Need to develop a database Costly (equipment) Not species-specific Not applicable on liquid samples and limited by the particle size (minimum 50 µm-100 µm) If sedimentation step previous to the NIRM analysis, use of organic solvents	Developments for mammalian versus non-mammalian identification
Near infrared spectroscopy (NIRS)	Identifies: – animal constituents in feedstuffs – fish versus terrestrial Existence of automated equipments (high throughput) Screening method Quantitative analysis LOD up to 1%	Need to develop a database Inaccurate, need for confirmatory method	Construction of large database Improvement of the LOD

in separation of the sediment into two parts. The amount of the bone sediment obtained after applying the French method is even smaller than that obtained with the standard sedimentation procedure, meaning that the results of the two methods are incomparable. Another advantage of sedimentation is the defatting of the material in the tetrachloroethylene solution, which results in a clear view of the particles.

Slides for the compound microscope, providing higher magnifications, are principally prepared in paraffin oil, although chemicals such as xylol and glycerol might yield comparable views. Additional reagents might be used, such as alizarin red (staining of bones), potassium hydroxide or sodium hydroxide (detection of muscles) and lead acetate (detection of keratin in hairs and horn). Several spot tests are also available for the identification of minerals (12, 79).

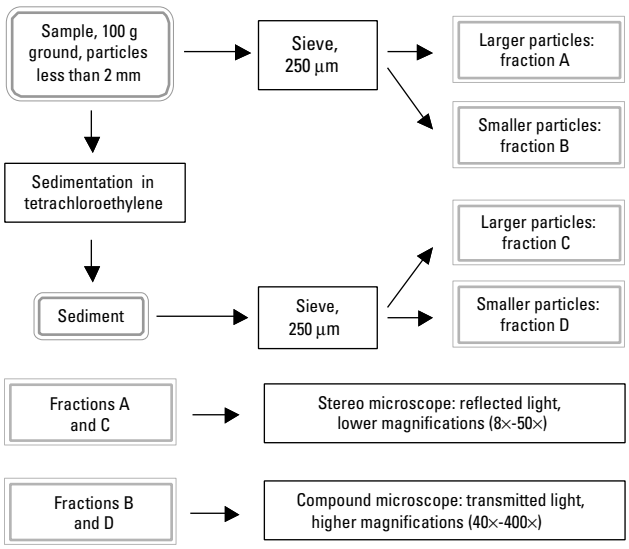


Fig. 1
Flow diagram of the procedure for microscopic examination

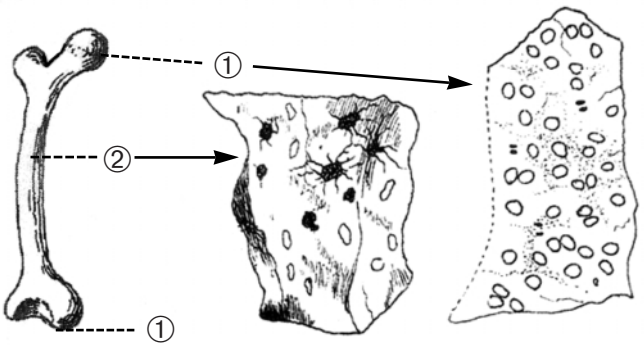
Characteristics for detection

The principal particles of animal origin that might be present in feeds are bones and muscle fibres. Additionally, cartilage, hairs, feather filaments, egg shells, fish scales and ligaments may also be present. Parts from organs, skin and other soft tissues are generally absent. Most of these particles show a limited number of characters. Bones appear to be the most persistent particles, even after the current EU rendering practice of sterilisation at 133°C and 3 bar for 20 min.

The structure of a typical long bone consists of a major central part (diaphysis) with terminal ends that are adapted for connection to other organs. The terminal ends also contain the growing segments (epiphyses). Sets of circular lamellae around central Haversian canals can be observed in the diaphysis. Lacunae containing bone cells (osteocytes) are organised along the lamellae, connected to each other and to the central canal by very fine webs of canaliculae.

The cells in cartilage (chondrocytes) form holes that are more globular in form. There is no canal structure in cartilage and cells are fed by diffusion (8, 19). Some of the specific histological features of cartilage can still be recognised in slaughter by-products, even after heat-treatment (Fig. 2). The following general descriptions refer to the different classes of vertebrates, i.e. mammal, avian (poultry) and fish.

At lower magnifications, the general appearance and colour of the sediment is the first indication of the origin of the MBM. Mammal bone particles are white or cream, whereas poultry bones generally have a darker colour and are more splintered and sharply pointed in shape. These bones are all opaque in contrast to fish bones, which are more transparent. The small, parallel-sided and hyaline fish bones can be seen in sediments of fish tissue.



① Epiphyse
② Diaphysis

Fig. 2
Different structures in bones from meat-and-bone meal originating from mammals
(adapted from Schweizer, 68)

Bone particles from mammals at higher magnifications show a more or less globular appearance with elliptical to almost globular lacunae. Canaliculae may be visible depending on the quality and opaqueness of the bone particle. The direction in circular lamellae is sometimes visible (Fig. 3a, Fig. 3b). Particles from poultry usually show a more splintered (sharp edged) appearance, which is caused by the different structure of the air filled bones. Lacunae are more globular and denser compared to mammal bone particles. Canaliculae are not visible (Fig. 3c). Fish bones often show parallel sides. Lacunae in fish bones are usually elongated with a clear fusiform net of canaliculae. There is a large diversity among fish species, i.e. in cod, the lacunae are linear without visible canaliculae (Fig. 3e, Fig. 3f).

These descriptions are very general and much variation can be observed. Cartilage structures of mammals, poultry and fish show mostly globular lacunae without connecting canals (Fig. 3g). Notwithstanding this appearance of cartilage, fish meal is usually distinguishable from slaughter by-products of land animals because of the typical shape and appearance of the lacunae and canaliculae. Differences between poultry and mammal meal are more difficult to detect and the range of characteristics may overlap (51, 52, 60). The characteristics provided are based on the examination of authentic samples of controlled origin and processing, and fit the information from histological textbooks (8, 19) and literature on animal meal (60, 68). Some of the more recent papers on the detection of animal meal in feeds present deviating information. Some or all of the characteristics of mammal and poultry bone particles appear to alternate between the two types (1, 43). European legislation does not make a distinction between different land animals and as a consequence of this, the mere presence of bone particles in feed is currently sufficient to reach a positive conclusion.

Both smooth and striated muscles are present in meal derived from mammals, poultry and fish (Fig. 3h). Muscle tissue is present as single fibres, which are broken to relatively short fragments. The width of the fibres depends largely on the state of nutrition of the animal and treatment during slaughter. The fibres are character-deficient and presence/absence is the most important conclusion for the detection of animal products in general.

Additional types of particles such as hairs, feather filaments (Fig. 3d), egg shells and fish scales (Fig. 3g) or fish gills (39) can help to confirm the presence of animal meal in feeds. Clearly, hairs will point to mammals, feather filaments and egg shells to avian material, and fish scales to fish as the source of contamination. Nevertheless, the presence of one of these fragments does not exclude the possibility of a mixture from more than one source. Although today, most rendering plants do have separated production lines for the different classes of vertebrates, carry-over or unintended contamination with mice or other animals cannot be excluded.

An attempt has been made to produce (semi-)quantitative results from microscopic analysis. According to EU guidelines (26), the amount of animal by-products (percentage) can be estimated according to the formula $(S \times c)/(W \times f) \times 100$, where:

S = sediment weight (mg)

c = correction factor (%) for the estimated portion of terrestrial animal bone constituents in the sediment

f = correction factor for the proportion of bone in the constituents of animal origin in the sample examined, and

W = weight of the sample material for the sedimentation (mg).

The correction factor c depends on the fraction of minerals or other particles in the sediment. In the guidelines, the assumption is made that 'if the type of animal meal present in the sample is known, it is possible to estimate the content'. Theoretical indications of the percentage of bone for bone meals is between 50% and 60% (f = 0.5 to 0.6) and in the case of meat meals, between 20% and 30% (f = 0.2 to 0.3) (28). In practice, much larger variation is found. As little as 10% of sediment can be found, even in the case of MBM. Large variations in the percentage of bones in fish meal are also observed (18).

Current situation and future developments

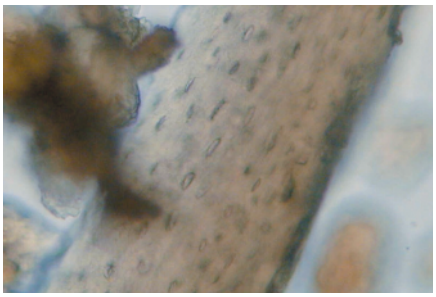
At present, meat meal, bone meal, MBM, spray-dried blood meal, poultry meal, fish meal and feather meal (whether hydrolysed or not) can be detected. However, detection does not mean that in all cases a reliable distinction can be made between the types of animal meal. Dicalcium phosphate can be detected and the source, either defatted bones or a mineral source, can be established in a majority of cases. Horn, grease, gelatine and plasma are difficult or impossible to detect when mixed in animal feeds. The lack of reliable distinction or detection possibilities is expressed in the legislation up to 2003 (31). As an example, since feather meal usually contains low amounts of bone particles, no distinction can be made between pure feather meal and mixtures of feather and poultry meal.

The improvement and further harmonisation of microscopic analysis of animal meal in feeds is part of a European project (3). The first part of this project is to develop sets of validated sample material of proven origin. These sets should include the effect of different rendering procedures, differences between geographical regions and should contain different combinations of contaminants at different concentrations. The current level of positive detection is at or below a concentration of 0.1% of MBM in animal feed. The detection limit might be influenced by the relative amount of bones in the MBM. The smaller the bone fraction, the smaller the amount of resulting sediment. Quantification of the MBM is possible in the samples prepared for this project because the original animal meal material is available so that f factors can be estimated accurately. The c factor needs to be estimated as well, which makes the results of the basic method and the French method for sedimentation virtually impossible to compare. Consequently, to achieve greater harmonisation of methods, the French method will not be used in the course of the European project. This means that all the sample sets prepared for the project will be able to be analysed quantitatively and inter-laboratory comparisons of the sedimentation procedure will be possible. However, quantitative analysis can still not be performed in routine control.

Another major point is the variation among different samples originating from each of the vertebrate classes, i.e. mammal, avian or fish. Colour characterisation at lower magnifications reveals considerable overlap between the classes, while higher magnification shows overlap in the shape of the lacunae and the visibility of the canaliculae (51, 52). This statement has two implications. Microscopists need to achieve a high level of expertise and the compilation of new and/or well-described characteristics is greatly recommended. The requirement for high-level expertise can be accommodated by the development of decision support systems (DSS). One of the deliverables of the European STRATFEED project will be a DSS dedicated to the identification of animal meal particles in feeds. The strength of the system will be the availability of several identification modules which will guide the user step-by-step through the process of identification of the sample under investigation. One of these modules consists of an identification key, which is a special form of a decision-tree. The system will also comprise a database (descriptions and pictures) with all the types of animal meal and their features, a gallery with photographs of samples from practice and a module with protocols and recipes of chemicals.

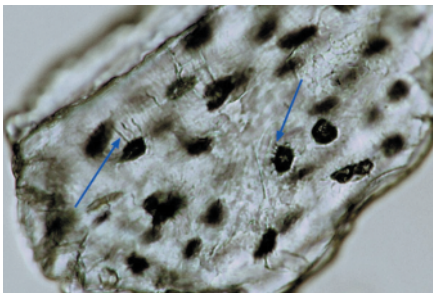
The recently approved intra-species recycling ban (36) requires reliable distinction between the different origins of animal meal

Mammal bone particles
a) Elliptical lacunae



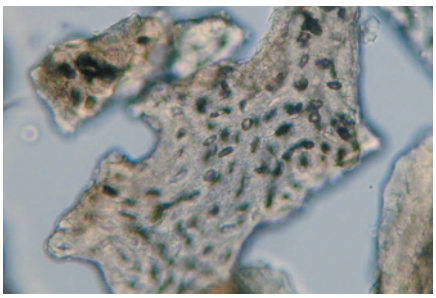
Courtesy: RIKILT, Wageningen, the Netherlands

Mammal bone particles
b) Faint canaliculae can be seen (arrows)



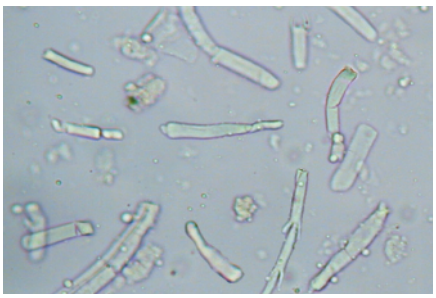
Courtesy: NUTRECO, Boxmeer, the Netherlands

Poultry particles
c) Bone particle with a dense pattern of almost spherical lacunae



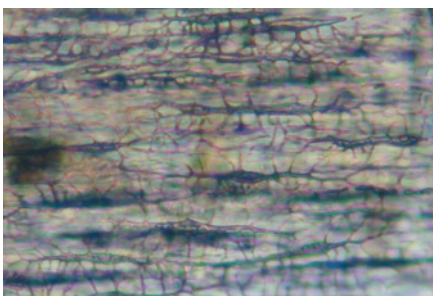
Courtesy: RIKILT, Wageningen, the Netherlands

Poultry particles
d) Set of feather filaments



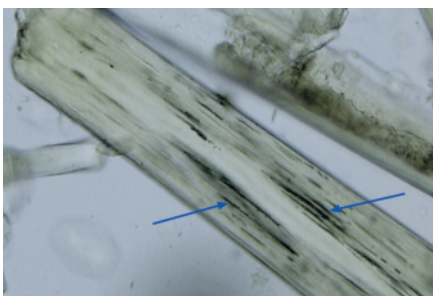
Courtesy: DPD, Lyngby, Denmark; LAGC, Barcelona, Spain

Fish bone particles
e) Herring with linear lacunae and fusiform canaliculae



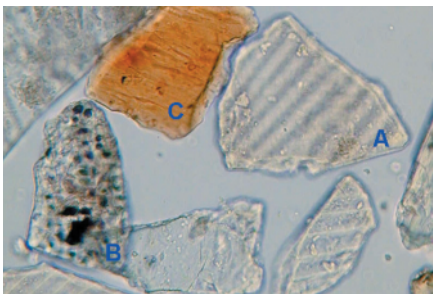
Courtesy: RIKILT, Wageningen, the Netherlands

Fish bone particles
f) Cod, lacunae (arrows) without canaliculae



Courtesy: RIKILT, Wageningen, the Netherlands

Fish particles
g) Fish scales (A), fish cartilage (B) and fat (C)



Courtesy: RIKILT, Wageningen, the Netherlands

Fish particles
h) Muscles, cross striated (arrow)

Courtesy: RAP, Posieux, Switzerland

Fig. 3
General appearance of particles in meat-and-bone meal

at the level of vertebrate classes, but also at higher taxonomy levels, i.e. bovine versus porcine. One of the goals of the STRATFEED project is to develop new characteristics and additional descriptions. In addition to bone fragments, other particles such as striated muscles might provide more information than that obtainable under current circumstances. Similarly, other methods such as PCR for deoxyribonucleic acid (DNA) detection, NIRS and near infrared microscopy (NIRM) might yield the necessary additional information. The foreseen DSS should also include information on these methods.

Near infrared spectroscopic methods

The principal interest in developing methods based on NIRS to manage the problem of accidental or deliberate addition of animal meal to feedstuffs is based on the fact that this is one of the most widely used analytical techniques in the feed sector (40, 41, 61, 67, 80). The principle of NIRS is based on absorption of light (absorbance) at selective wavelengths of the electromagnetic spectrum by the molecules constituting the analysed samples. The advantages of the NIRS technique are mainly rapidity, use of no dangerous reagents, minimal sample preparation, non-destructive analysis, economic attractiveness, good and repeatable signal intensity, as well as the potential to develop and commercialise a 'push-button' dedicated instrument (50). The main drawback of the NIRS technique is that the method is indirect and therefore requires large numbers of reference values on authenticated samples to form a calibration or discriminant model (11, 72). As NIRS is already widely used for quality control analysis in the feed industry, adoption of the method as an MBM screening device could be easily implemented in feed mills.

Near infrared spectroscopy

Figure 4 presents the near infrared spectra of feed materials of animal origin. Near infrared spectra contain mainly bands characteristic of the major compounds water, protein, fat and carbohydrates.

Near infrared spectroscopy is routinely used to determine chemical (i.e. water, protein, fat, ash, sugar, starch and fibre) and biological (i.e. digestibility and energy values) parameters in animal feeding and production (41, 61, 75). Recently, the near infrared signature of a sample was proposed for the detection and quantification of MBM in feed ingredients and feedstuffs. Garrido-Varo and Fernandez reported the results of a tentative study conducted to evaluate the potential of the NIRS technique (42). The study used samples spiked at different levels (0.5%-4%) and real samples (cattle and pig feeds) obtained from the feed sector. The authors concluded that NIRS could be used for instant detection and quantification of MBM at low levels. In 2001, Murray *et al.* demonstrated the use of NIRS for detecting MBM in fish meal (62). The study

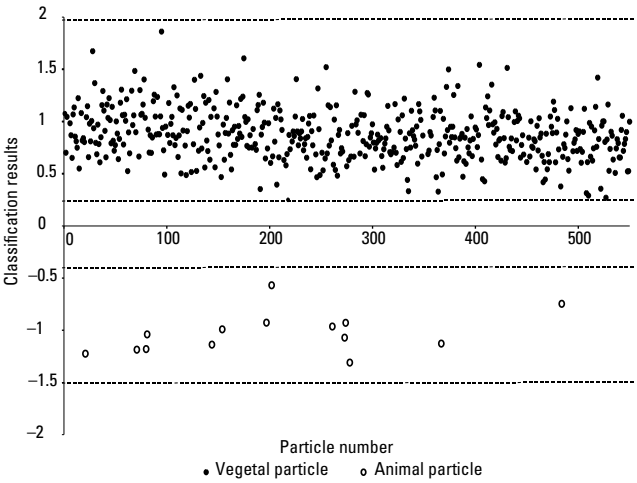


Fig. 4
Near infrared spectra of ground feed materials of animal origin
(spectra obtained on a Foss near infrared microscopy system 6,500)

involved testing some 46 pure fish meal samples and 90 fish meal samples spiked at 3%, 6% and 9% with MBM, and underlined the potential of NIRS to differentiate between animal protein from two different species.

One of the main features of the NIRS method is the possibility to analyse a large quantity of specimen. Indeed, analysis is performed on 10 g to 100 g of sample, allowing the natural inhomogeneity of feed samples to be taken into account and reducing the probability of false-negatives resulting from inappropriate sampling procedures (37). Efforts have to be made to compile a spectral library as large as possible and robust mathematical models have to be created to determine the exact LOD of the technique and establish the potential of the method to discriminate between animal ingredients from different sources. However, the use of spectral libraries at national or European level for other countries, as well as the percentage of false-positives and false-negatives have not yet been reported (3). At present, the major drawback of the NIRS technique is that the LOD is higher than 1% and the method cannot be used alone as legal evidence. To try to overcome this limitation, other NIR techniques, such as NIRM and a NIR camera have been proposed in the BSE research programme. Nevertheless NIRS may provide screening as the first line of defence of the food chain and enable more costly methods to be used more productively on suspect specimens.

Near infrared microscopy and near infrared camera

The above-mentioned studies concern the application of the so-called 'macro' NIR spectroscopic technique, with which a single spectrum is obtained from the analysis of one specimen. With the NIR microscope and NIR camera, also called 'micro' NIR instruments, several spectra (up to hundreds or thousands) can be obtained from the analysis of one specimen (11). The NIR microscope combines the analytical advantages of microscopy

and spectroscopy techniques (9). Briefly, with this type of instrument, the infrared beam is focused, using a dedicated microscope, on each particle of a sample and the NIR spectrum is collected. Sample analysis results in the collection of hundreds of spectra, each one being the molecular signature of a particle from one of the feed ingredients used in the formulation of the compound feed.

Piraux and Darenne (66) and Baeten *et al.* (10) have demonstrated the potential of the NIR microscopic technique in the detection and quantification of MBM in feedstuffs. They constructed spectral libraries including thousands of spectra of single particles from animal, vegetal and mineral feed ingredients and used these to identify the origin of unknown particles. Several clean and adulterated feeds were analysed by NIR microscopy. All adulterated samples were identified as such and quantified, calculated on the basis of the number of particles detected as animal over the total number of particles analysed. None of the clean samples were declared adulterated. Figure 5 presents the analytical results obtained for a sample spiked with 3% MBM. The figure shows the result of an equation that allows for discrimination between animal and vegetal particles. Each filled circle represents the particles classified as vegetal, while the open circles are the particles classified as animal. The dashed lines are the 95% confidence intervals of each group, calculated on the calibration samples (10, 66).

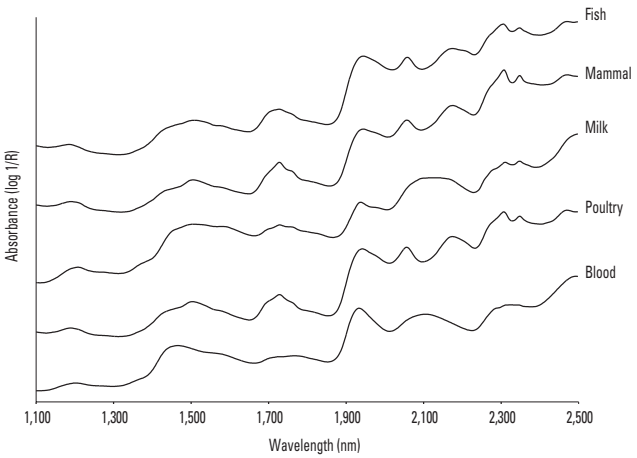


Fig. 5
Results of the near infrared microscopy analysis of a sample containing 3% meat-and-bone meal
Source: Agricultural Research Centre of Gembloux (GRAGx), Belgium

As in classical microscopy, the LOD of NIRM depends on the number of particles analysed. Statistics indicate that to detect at least one MBM particle with 95% certainty in a 0.1% adulterated feed, 3,000 particles have to be analysed. A recently undertaken study (A.-M. Michotte-Renier, V. Baeten and P. Dardenne, unpublished results) demonstrated that adulteration can be detected at levels as low as 0.1% following analysis of about 3,000 particles. However, analysis of 3,000 particles takes several days, which is unrealistic. To overcome this limitation, two strategies have been proposed. The first is to add a preliminary sedimentation step to the NIRM analysis. The procedure followed is exactly the same as that presented above for classical microscopy, but the eyes and expertise of the microscopist are replaced by an infrared detector and discrimination equations.

Figure 6 shows the results of NIRM detection for a series of samples spiked with MBM. The NIRM analysis of one sample takes about one hour. The *f* factor of the MBM sample was known (*f* = 0.20). This diagram shows that adulteration can be detected at a level as low as 0.05% using the NIRM method (including a sedimentation step).

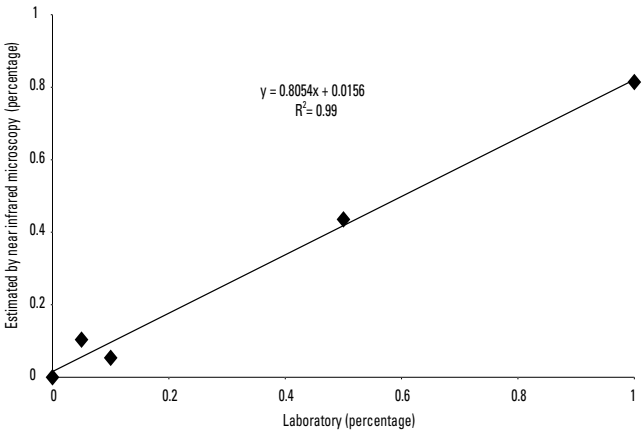


Fig. 6
Results of the near infrared microscopy analysis of the sedimentation fraction of five spiked samples
Source: Agricultural Research Centre of Gembloux (GRAGx), Belgium

The second strategy to boost NIR analysis of particles is by using a NIR camera (11, 58). A NIR camera (also called a NIR imaging system) takes sequential pictures of a pre-defined sample area at different wavelengths. The pictures are collected in a three-dimensional matrix, the first two dimensions yielding an image and the third dimension giving a spectrum for each pixel of the sample area analysed. This type of instrument allows the analysis of about 500 particles in 5 min. Figure 7 shows the picture obtained at 1,470 nm for an intentional positive sample. Quantitative analysis can be performed by calculating the ratio of the area corresponding to the MBM particles over the area covered by all the particles. Simultaneous analysis of hundreds or thousands of spectra using a NIR imaging system has the advantages of rapidity, sensitivity and specificity and is an attractive way of controlling the correct application of the ban on the use of animal meal (V. Baeten, A.-M. Michotte-Renier and P. Dardenne, unpublished results).

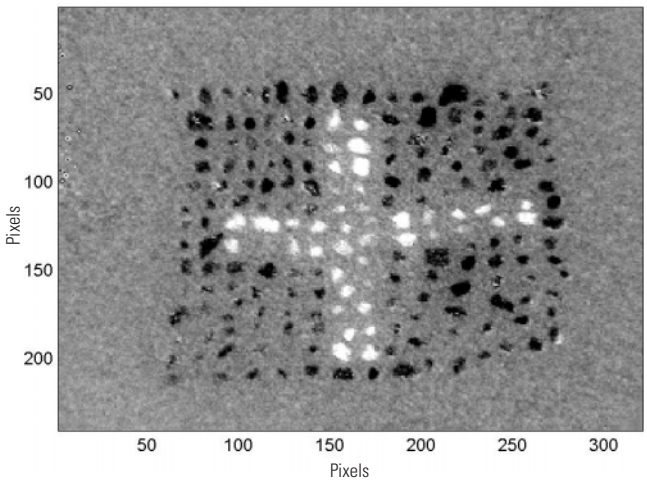


Fig. 7
Results of the analysis of an intentional positive sample using a near infrared camera
Picture of the absorbances scale at 1,470 nm
Source: Agricultural Research Centre of Gembloux (GRAGx), Belgium

General comments and future developments

Methods based on NIRS have their place in the global strategy to assess the control of feed ingredients and feedstuffs. The methods can be managed easily and could be used to rapidly detect suspect materials and provide a 'provisional declaration' for the gross volume of material traded. The suspect materials would then have to be analysed using a forensic method to give final confirmation (41). The macro NIRS method could be the first line of defence against accidental contamination (i.e. cross-contamination) and fraudulent practice. The methods could be used as part of the routine quality control procedure and would restrict the use of more costly tests only to suspect samples (62). Current research on micro NIRS methods (i.e. NIRM and NIR camera) clearly demonstrates the great potential of these methods to detect and quantify low levels of added MBM. The decisive advantage is that all the constituents of the MBM are taken into account (bones, muscles, etc.) and the methods can be easily adapted to identify and quantify, in a single analysis, a wide range of animal feed ingredients (i.e. fish, blood, milk by-products, feathers) as well as vegetal ingredients.

Molecular biology techniques for the detection of animal by-products

There are two major analyses on which molecular biology detection techniques can be focused, i.e. proteins and nucleic acids. These compounds are always present in rendered animal by-products, but detection depends on how the assays are conducted and on the previous thermal treatment imposed.

Immunochemical methods

Meat speciation based on specific recognition through the antibody-antigen affinity has been applied for a long time (46) and several commercial kits have been developed for the determination of raw and moderately cooked meat in food. In 1995, Hofmann *et al.* reported on the application of a commercial ELISA kit for the analysis of feed samples, demonstrating that MBM cannot be detected in feed that has been previously sterilised at elevated temperatures (above 120°C) (48). These results therefore hint at an intrinsic problem of immunoassays when applied to the analysis of feed containing material treated at high temperatures. However, the study (47, 48) showed that the strong dependence of the response of immunoassay on the sterilisation temperature allowed the test kit to be used for verifying if the products of rendering plants had been processed at the temperatures demanded by European legislation (29). Based on these results, von Holst *et al.* (77) and Pallaroni *et al.* (65) performed extensive investigations to establish how strongly the result of the immunoassay depends on the various sterilisation conditions of the MBM. This work was conducted to examine the reliability of the immunoassay technique when applied to the control of rendering processes. Nevertheless, the research also had implications for the subject of this chapter since the application of molecular biology methods to the proof of the presence of animal tissues requires that the target analytes (a specific protein or a DNA sequence) can be detected irrespective of the heat-treatment to which the MBM was subjected.

Pallaroni *et al.* (65) and von Holst *et al.* (77) evaluated the impact of sterilisation factors such as temperature, pressure and duration on the response of commercial immunoassay tests for beef and pork. The studies showed that, in both cases, the analysis of MBM gave elevated immunoassay responses when at least one of the criteria (i.e. temperature) did not fulfil the requirements of the European legislation (i.e. at least 133°C, 3 bar for a minimum of 20 min on particles not exceeding 50 mm in size). On the other hand, the results showed that adulteration of feed with MBM cannot be proved if the MBM was properly sterilised.

However, the animal species used in animal by-products may seemingly still be identified with well-designed antibodies raised against thermo-stable antigens. Kang'ethe and Gathuma (54) were able to identify the animal species present in autoclaved meat mixes, albeit with some cross-reactivity between species. The existence of such species-specific thermo-stable epitopes on proteins in animal tissues had in fact been known for some years (17, 45, 59).

Ansfield (5) was the first to explore the possibility of raising antibodies against thermo-stable antigens that are assumed to withstand severe rendering processes and to use them to check animal species. This led to a patented and in-house validated

immunoassay (6, 7), reputed to be applicable to ruminant and porcine proteins in compound animal feedstuffs. The method consists of a specifically dedicated extraction technique involving heat-treatment, followed by a two-step ammonium sulphate precipitation procedure, the first step for removal of interfering proteins, such as gelatine, that could affect the immunological recognition, and the second step for concentrating the target proteins on which a double sandwich ELISA is applied. This procedure is far more complex when used for compound animal feed rather than pure MBM and requires many precautions (i.e. an additional heat-treatment step to suppress the effects of oils and fats) to limit false-positives and false-negatives as much as possible (possibly due to interactions with plant proteins or matrix-induced loss of signal). Nevertheless, the method presents pitfalls for routine application at least (4). It is also worth noting that although the assay generally results in negative responses when applied to milk or blood products, positive signals are occasionally obtained.

A Danish company developed antibodies that were raised against heat-treated albumin which proved to be effective for species recognition, even on steam-treated MBM which reached temperatures largely above 133°C, but the behaviour of these antibodies against compound feed has not yet been reported. Similarly, in Spring 2002, an American company marketed a lateral flow assay targeting a heat-stable muscle protein (troponin I), making it possible to ascertain the presence of ruminant material in MBM at a detection limit of 5%. Using the latter strip-test, the authors found evidence of the presence of ruminant material in MBM containing 50% of bovine material having endured a batch process at up to 141°C for 20 min. (O. Fumière *et al.*, unpublished results). The same company has produced another test of this type, but which is applicable to feed, with a claimed detection limit of 1%.

Immunoassays are generally rapid and easy to perform as they do not require highly trained staff. For instance, the strip-test cited above only requires boiling the meal for 10 min. in a buffer solution and then dropping the strip into an aliquot of this solution and waiting for 10 min. before reading the strip. Research is probably still required to obtain the most effective antibodies and to solve the problems related to the extraction of proteins from compound feed.

In conclusion, immunochemical assays can be considered as screening methods in terms of their practicability, if sensitivity and specificity requirements are fulfilled (i.e. no false-negative results and only a limited percentage of false-positives). Nevertheless, other more specific techniques need to be applied to those samples found to be positive to confirm the forensic validity of the results.

Molecular genetic methods

State-of-the art techniques before application of real-time polymerase chain reaction

Identifying animal taxons through nucleic acids in meat products was first achieved by simple DNA-DNA hybridisation reactions, generally in a type of dot blot design on filters, using labelled DNA probes (13, 15, 21, 22, 81). Some of these experiments did focus on cooked or canned meat and produced successful results, with probes being either species-specific DNA segments or randomly obtained fragments from the entire genome. With the development of genetic amplification methods, mainly the PCR, only those methods capable of detecting a well-specified target sequence by amplification thereof are now considered for DNA-based food/feed component analysis (57) as they are much more sensitive.

Tartaglia *et al.* (73) was the first to design a PCR test enabling detection of ruminant material in MBM, even when incorporated in feed. The chosen target was a mitochondrial DNA segment of 271 bp covering the ATPase subunit 8 gene and overlapping portions of $tRNA^{Lys}$ and ATPase subunit 6. An interesting feature of this target is that this $tRNA^{Lys}$–ATPase8–ATPase6 gene configuration is not found in the mitochondrial genomes of higher plants. The authors of this first PCR-based protocol showed that 0.125% of MBM was still detectable on DNA obtained with a silica-based extraction protocol (73). An inter-laboratory study with this PCR method was conducted in 1999 by the Joint Research Centre (JRC) of the EC (76). Twenty-four laboratories participated in this study, analysing compound feed that contained MBM at various concentration levels, all using the Tartaglia method. Prior to the validation study, the participants attended a training course to become acquainted with the method. The aim of the study was to examine whether the proposed method could be used to detect MBM in compound feed at a concentration of about 0.5% or lower. In particular, interest lay in establishing the portion of false-negative results at the target concentration level and the portion of false-positive results of the blank material.

The main characteristics of the test materials are summarised below, as follows:

– five concentration levels (0%, 0.1%, 0.5%, 1% and 2%) of MBM, containing about 50% bovine material, produced in a commercial batch-type rendering plant (sterilisation temperature of about 134°C to 135°C)

– two different compound feed matrices prepared at laboratory level using, for each mix, six typical vegetal ingredients according to realistic recipes. In this way, the risk of cross-contamination with traces of bovine tissue was minimised compared with the use of commercial compound feed

– ten different test materials (5 concentration levels × 2 feed matrices) were sent out as blind duplicates to the laboratories for analysis.

Statistical evaluation of the results demonstrated that the PCR method assessed in this study was not fit for the purpose. The method yielded an unacceptably high portion of false-negatives (above 20%), irrespective of the MBM concentration and the feed material analysed. In addition, an even higher number of false-positive results were observed for the blank material. Since the participants provided observations on the protocol, some reasons for the failure of the study could be indicated. Problems could be associated with the extraction/clean-up procedure leading to occasional inhibition of the polymerase. It was also pointed out that the target DNA sequence in MBM treated at high temperatures might be degraded.

Five other articles with classical PCR (i.e. with agarose gel electrophoresis for analysis of PCR products) were published on this topic, but the usefulness of PCR for MBM detection is unclear based on these data. In the first study, Wang *et al.* (78) used the Tartaglia method but with another DNA extraction technique on feed. They demonstrated that more rapid and reliable results could be obtained using their method compared to the original one. This suggests that the problems of the JRC ring trial might have been linked to extraction, and more specifically, to the purity of the extract with regard to PCR inhibitors, which, of course, might be highly matrix-dependent. However, as noted later in this discussion, this was not the case.

Recently the same team organised a validation study with four laboratories, co-ordinated by the United States Food and Drug Administration (63). In this study, they used the Tartaglia method, even for the extraction protocol, with slight modifications regarding the lysis buffer. Surprisingly, a very low rate of false-negative results (1.67%) was observed, even at a concentration level of 0.125% MBM in the feed. However, the results of this paper are not directly comparable with those of the former JRC study since the sterilisation conditions of the MBM were not specified and the material may have been processed less severely than when applying current European rendering standards.

Two other articles focused on the use of targets based on the mitochondrial-encoded cytochrome b gene, but yielded completely divergent results. In the first study, Kingombe *et al.* (55) concluded that on properly rendered material, detection of bovine DNA segments by PCR fails because the DNA is too fragmented. They used two targets (274 bp and 464 bp) and a standard, resin-based DNA extraction protocol. Conversely, in the second study, Bellagamba *et al.* (14) described successful application of PCR-restriction fragment length polymorphism (RFLP) for identification of the animal species present in MBM contained in feed. The purpose of PCR-RFLP is to amplify a common fragment in several species by use of universal primers (i.e. primers suitable for a group of animals because their sequences correspond to highly conserved segments) and then to try to distinguish the respective origins of the fragments with their internal differences evidenced from restriction patterns with several type II endonucleases. Applying this PCR-RFLP assay with a target of 359 bp, Bellagamba *et al.* (14) were able to detect and identify an interesting range of animal species (cattle, buffalo, sheep, goat, horse, pig, chicken and turkey) contained in poultry meal and in MBM samples that were, unfortunately, very scantily described. Moreover, the technique was also shown to be efficient for feedstuffs with 0.5% of poultry meal or meat meal.

Finally Colgan *et al.* (16) extended the use of the mitochondrial $tRNA^{Lys}$–ATPase8–ATPase6 target designed for cattle by Tartaglia *et al.* (73) to poultry, porcine and ovine DNA. The sizes of these targets ranged between 250 bp and 350 bp. Two DNA extraction techniques were tested. The authors found that on some of the pure MBM samples, none of the analysed species could be detected by PCR. They established that inhibition was not responsible for this and concluded that the genetic material was too severely degraded thermally to be suitable for PCR.

Meat-and-bone meal detection with real-time polymerase chain reaction

To determine whether PCR is suitable as a detection technique on properly heat-treated MBM, five MBM samples were produced under wet sterilisation in an industrial batch-type rendering plant at different temperatures, measured with an internal temperature probe as 125°C, 130°C, 135°C, 138°C and 141°C. It was considered that the DNA in this material would be highly degraded and that therefore very small targets had to be selected (20). Mitochondrial-encoded targets of sizes ranging from 60 bp to 70 bp were carefully selected for several species (bovine, porcine, ovine and equine targets) or animal groups (fish) (M. Dubois *et al.*, unpublished results), and because of their extremely small size, the PCR format was performed in real time in order to avoid agarose gel electrophoresis which is inconvenient on such small fragments (M. Dubois *et al.*, unpublished results). Real-time PCR was achieved with appropriate Taqman probes (M. Dubois *et al.*, unpublished results). With this type of real-time PCR format, the production of amplicon during the entire amplification reaction can be followed by measuring light emission that occurs only when the Taqman probe to which a fluorescent dye is attached recognises a complementary strand (44). The specificity of this PCR format is greater than in classical PCR because the measured signal is not only dependent on the chosen primers, but a second selectivity step is introduced with the probe generating the fluorescent signal. This real-time PCR protocol with small bovine and porcine targets was applied to the five above-mentioned MBM samples produced at the given temperatures. The DNA of both species was clearly detectable in all samples, demonstrating that with the considered MBM

production process, irrespective of the temperature up to 141°C (Fig. 8), sufficient DNA was present to perform PCR. However, detection failed for all samples (M. Dubois *et al.*, unpublished results) when target sizes reached 275 bp and 350 bp, i.e. about the sizes of the targets used by Tartaglia *et al.* (73) and Bellagamba *et al.* (14). In addition to the very small size requirement that is clearly demonstrated by this experiment, it is also of the utmost importance to possess a large copy number of targets such as the mitochondrial targets. However, nuclear targets such as short interspersed nuclear elements (SINE) (38, 56) are also suitable (H. Aarts *et al.*, unpublished results), although apparently less likely to have another essential feature of properly set-up PCR tests, namely to generate no signal at all with the negative controls (no template or extraction control).

Once it was firmly established that PCR could be used to detect animal DNA segments in MBM produced in conformity with EU requirements, tests were extended to detection in compound feed. For this, twenty blind samples from the above-mentioned JRC ring trial were assayed. Even if results were not entirely conclusive, most samples were nevertheless correctly classified as negative or positive (with the porcine probe) and even some ranking according to spiking level was possible (M. Dubois *et al.*, unpublished results). Taking into account that species composition in these samples was almost 50% pig and 50% cattle and that the 0.1% MBM level was correctly detected, in most cases, the LOD per species may be concluded to be about 0.05%. However, this detection limit may vary according to the production process for MBM and to make a more general statement about the detection limits achievable by PCR, an overview of all possible production processes has to be conducted.

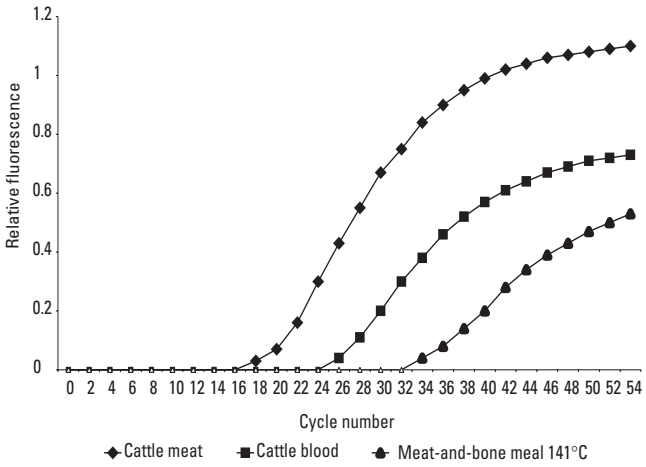


Fig. 8
Results of a real-time polymerase chain reaction assay
The curves are obtained through measurement of a fluorescent signal (y-axis gives intensity of fluorescence) that represents the production of a specific amplification product. This represents a real-time reaction as the measurement performed is over all the cycles (given along the x-axis) of the reaction in contrast to classical polymerase chain reaction which performs an end-point detection. The later a signal appears during the reaction with a time-scale measured in cycles, the less target is present in the reaction vial. The three amplification curves shown were generated with a target/probe (Taqman probe) configuration specific for cattle on deoxyribonucleic acid (DNA) extracts from beef meat, cattle blood and meat-and-bone meal (MBM). The MBM contained 40% to 60% cattle material and was processed at 141°C. Since the rendering process damaged a large proportion of the sought DNA target in the MBM sample, the signal appears much later than with fresh meat or blood. However, the presence of cattle DNA in the MBM sample can still clearly be demonstrated by this assay. In the same experiment, other DNA extracts (from pig, chicken, turkey, salmon, fish meal and barley) were tested together with a no-template control (i.e. simply water) and gave either no signal at all or a faint and non-significant background noise signal as can be seen on the left side of the diagram

Advantages and drawbacks of polymerase chain reaction

At temperatures higher than those tested for the production process of MBM, a threshold will be reached above which PCR is no longer of use. This limit probably varies according to the type of process. This drawback is rather theoretical as economic (energy costs) and nutritional (destruction of some amino acids) reasons prevent renderers greatly exceeding the legal minimum requirements for sterilisation temperature. Furthermore, pressure security limits of most existing sterilisation devices are such that temperatures above 133°C are confined to a small number of plants.

Apart perhaps from the presence of some inhibitory compounds, PCR is largely independent of matrix formulation. The technique can be applied to liquids, on samples ground down to very tiny particles and on animal by-products devoid of bones. In addition to this flexibility, the chief value of PCR is the potential of the method for making clear identifications, not only at species level but also at higher taxon levels (i.e. ruminants or mammalian). This will be of great interest when MBM is once again authorised in feed, but with appropriate measures to prevent 'cannibalism' (ban on intra-species recycling).

Among the drawbacks of PCR is the continuous attention that is required to prevent contamination. Furthermore, the technique only allows indication of possible sources of animal DNA. The link between MBM presence and a positive signal is not at all straightforward because milk, blood and even fat may be potential sources of trace animal DNA. Sedimentation steps included prior to DNA extraction might solve this type of interference (G. Vaccari and B. Chiappini, unpublished results), but this requires more in-depth investigation. Polymerase chain

reaction is also a relatively costly method, not only in regard to the reagents but also regarding equipment, particularly when a real-time thermocycler is required.

The main characteristics of methods using immunoassays compared to PCR are summarised in Table III.

Future developments

In so far as merely qualitative assays are concerned, the detection of the presence of several species in the same vial will be possible by real-time PCR using several fluorescent dyes and silent quenchers for the probes.

Polymerase chain reaction is thought to be effective for products resulting from most rendering processes, at least on pure MBM produced under normal conditions according to legal requirements. Conversely, whether detection of relatively high levels of MBM in compound feed will always be achievable is far from clear, as this might depend largely on how the MBM was produced. An overview of the principal processes used throughout the world therefore appears to be essential and is ongoing (G. Vaccari and B. Chiappini, unpublished results). The diversity of production processes leading to very different degradation levels of genetic material seems to have been totally overlooked in the development of the methods by Tartaglia *et al.* (73) and Bellagamba *et al.* (14). For the same reason, it seems unlikely that the following will be developed:

– a PCR test that could check whether the correct heat-treatment has been applied without any prior knowledge of the type of rendering process or

– quantitative determinations, even if, as noted for the JRC ring trial, the real-time PCR technique offers some potential when the same MBM is used at different levels.

The only possibility that remains in this regard is to verify whether a link exists between the size distribution of fragments and the rate of loss of material that would be process-independent, because then development of quantitative assays using PCR would not be impossible using current technology.

Conclusions

Many countries, and especially the EU, have introduced various laws to ensure the safety of feed in terms of BSE. In principle, the major objective of this legislation is to restrict the use of animal by-products as ingredients for the production of compound feed. The bans on feeding MBM to ruminants and on intra-species recycling ('cannibalism') are central measures in the risk reduction strategy for BSE. Enforcing such regulations requires validated tests capable of recognising the presence and species of animal by-products in compound animal feeds.

This paper introduced the different methods currently applied to the analysis of feed, which are microscopy, PCR, immunoassay and spectroscopy. Comparing the respective performance characteristics revealed that there is no ultimate approach that would fulfil all requirements. When evaluating the performance of a method, taking into account the purpose of the analyses is also important. For instance, enforcing the

Table III
Comparison of polymerase chain reaction and immunoassay

Feature	Immunoassay	Polymerase chain reaction
Requires confined rooms	No	Yes
Requires relatively expensive apparatus and reagents	No	Yes
Sample pre-treatment	Relatively time-consuming	Can be automated
Detection limit on feeds	1%	0.1%
Sensitivity and specificity	False-negative results sometimes obtained due to the presence of gelatine and fats in the sample	False-negative results sometimes obtained due to the presence of inhibitors in those extracts with high deoxyribonucleic acid concentration
	False-positive results sometimes obtained due to cross-reactivities with plant proteins	No false-positive results
Forensic validity	No	Yes
Others	Possibility to use the assay to check for appropriate heat-treatments of meat-and-bone meals	Opportunity to modulate the assay according to different taxon specificities: vertebrates, mammalian, ruminant
	Requires laboratory animals to produce antibodies	Possibility to use the test on other ruminant constituents, the use of which is restricted in feeds (i.e. raw tallow)
	Standardisation affected by the quality of antibodies as biological products	Flexible approach according to legislative updates
		Laboratory animals not required
		Easy standardisation of reagents

ban of MBM in ruminant feed would require a method that allows the determination of mammalian tissue in feed, whereas the prohibition of intra-species recycling would definitely require the ability to perform a species-specific analysis.

At present, feed microscopy is the only official method for the detection of MBM in feed in the EU. Based predominantly on the presence of bones, the method is not affected by heat-treatment applied during rendering. However, disposing of a suitable technique for routine control requires improving and harmonising microscopy, particularly with regard to differentiation, not only between classes of vertebrates, but also at higher taxonomy levels.

When compared to microscopy, NIRS is certainly more suitable for large screening applications in terms of sample output and automation, particularly because databases with reference values are becoming increasingly available. However, for the detection of MBM in feeds, spectroscopy methods still have a ten-fold higher LOD than microscopy (1% versus 0.1%) which therefore needs to be combined with confirmatory techniques. Interesting developments will be provided by NIRM and the NIR camera. By associating the advantages of microscopy and spectroscopy, these methods can achieve low LODs (0.1%), resulting in far more precise quantitation. Further improvements are being achieved, reducing the analysis of samples from several days to a few minutes. The high costs of NIR equipment and difficulty in recognising animal groups or species must however be taken into consideration.

In contrast to the previous techniques, the application of immunochemical and molecular methods is limited by heat-treatment of the samples. While these techniques are reliable and precise with moderately heat-treated materials (i.e. at least up to 135°C, even with wet sterilisation), when severe sterilising conditions are applied, performances become poor. Various companies and institutes are trying to overcome the limitation due to heat-treatment through substantial research efforts and, consequently, rapid development can be observed in this area. Considerable progress in the determination of the presence of MBM in animal feed and in the recognition of the species involved has been achieved with PCR. The technique has been demonstrated to tolerate temperatures of up to 141°C whilst differentiating between the animal species present in compound feed.

Following examination of the various methods presented in this paper, significant improvement is clearly being achieved with all the different techniques. Assessing the advantages and disadvantages of the different approaches also showed that the methods are complementary rather than competitive. For instance, spectroscopy and immunoassays lend themselves well as screening methods, whereas results from microscopy and PCR have a high forensic value. As another example, microscopy is practically not affected by the sterilisation conditions of MBM, which is not the case for PCR and immunoassay. Current ongoing improvement of all the methods will finally allow control laboratories to single out the appropriate methodology, which could also be a combination of different methods, for a specific analytical purpose.

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Aperçu des méthodes de détection des tissus animaux dans les aliments pour animaux, introduites en réponse aux préoccupations de santé publique liées à l'encéphalopathie spongiforme bovine

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Résumé

La mise en application de l'interdiction des farines de viande et d'os dans les aliments destinés aux animaux d'élevage, notamment aux ruminants, est considérée comme une mesure importante pour empêcher la propagation de l'encéphalopathie spongiforme bovine. Les auteurs décrivent les méthodes d'analyse employées actuellement pour la détection et l'identification des tissus animaux dans les aliments pour animaux. De plus, ils passent en revue les prescriptions récemment approuvées, telles que l'interdiction du recyclage au sein de l'espèce (pratique consistant à nourrir des animaux à l'aide de protéines issues de tout ou partie de l'organisme de sujets de la même espèce). Quatre approches différentes seraient aujourd'hui privilégiées, à savoir l'analyse microscopique, l'amplification en chaîne par polymérase, l'analyse immunologique et la spectroscopie/microscopie dans le proche infrarouge. Les auteurs présentent et comparent les principales caractéristiques de ces méthodes en termes de performances, de même que leurs avantages et inconvénients spécifiques. Ils accordent également une attention particulière à l'impact des conditions d'équarrissage, notamment au traitement à température élevée, ainsi qu'à l'utilisation des techniques de biologie moléculaire.

Mots-clés

Aliment pour animaux – Amplification en chaîne par polymérase – Encéphalopathie spongiforme bovine – Farine de viande et d'os – Identification des espèces – Législation – Méthode immunologique – Microscopie – Spectroscopie dans le proche infrarouge.

■

Panorámica general de las pruebas de detección de tejidos animales en piensos que se están aplicando en respuesta a las preocupaciones de salud pública ligadas a la encefalopatía espongiforme bovina

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Resumen

Para prevenir la propagación de la encefalopatía espongiforme bovina se considera importante velar por el cumplimiento de la prohibición de introducir harinas de carne y huesos en los piensos para animales de granja, en especial rumiantes. Los autores describen los métodos analíticos utilizados actualmente para detectar y reconocer tejidos animales en los piensos. También exponen los requisitos impuestos de un tiempo a esta parte, por ejemplo la prohibición del

reciclaje intraespecífico (procedimiento consistente en alimentar a una especie animal con proteínas derivadas del cuerpo o parte del cuerpo de otros ejemplares de la misma especie). Hoy en día se utilizan en principio cuatro tipos de métodos distintos: análisis microscópico, reacción en cadena de la polimerasa, análisis por inmunoensayo y espectroscopia/microscopía de infrarrojo cercano. Los autores exponen y comparan los principales parámetros de rendimiento de esos métodos, señalando las ventajas e inconvenientes de cada uno de ellos. También examinan con especial atención el impacto de las condiciones de procesamiento técnico, en particular las temperaturas elevadas, y el uso de técnicas de biología molecular.

Palabras clave
Espectroscopia de infrarrojo cercano – Harina de carne y huesos – Legislación – Microscopía – Pienso animal – Reacción en cadena de la polimerasa.

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