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New developments in the detection and identification of processed animal proteins in feeds

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Abstract

It is generally accepted that the most likely route of infection of cattle with bovine spongiform encephalopathy (BSE) is by consumption of feeds containing low levels of processed animal proteins (PAPs). This likely route of infection resulted in feed bans, which were primarily aimed at ruminant feeds, and were later extended to all feeds for farmed animals. The feed bans were expected to develop into a future enforcement of the "species-to-species" ban, which prohibits only the feeding of animal-specific proteins to the same species. The species-to-species ban requires support of species-specific identification methods.

In the European Union, microscopic evaluation is currently the only accepted method for the detection and characterization of PAPs in feeds, since it is possible to detect contaminations at the requested level of 1 g/kg with hardly any false negative nor positive results. This method is predominantly focused on the presence and characteristics of bone fragments, although other structures, *e.g.* muscle fibres, may provide circumstantial evidence of the respective animal types. Recent developments are the identification of bone fragments at the level of classes (mammal *versus* bird *versus* fish), supported by image analysis of bone characteristics.

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Detection of DNA and specific proteins are additional methods that can be applied for the identification of PAPs in feeds. DNA is known to be very specific for animal species and breeds, whereas proteins can also indicate the type of tissue. The latter aspect is important to differentiate between proteins that are authorised in animal nutrition from banned proteins. Improvements can be noted in recent years for both methods. For a proper application of polymerase chain reaction (PCR) to detect specific sequences of DNA, primer sets have been developed which amplify a DNA sequence shorter than approximately 100 nucleotides. Specific antibodies have been developed for protein detection of ruminant or bovine material. Recent results of various studies indicate that specific DNA and protein detection methods can detect PAPs at a contamination level of 1 g/kg. However, full validation of these methods still needs to be carried out.

Other methods such as near-infrared spectroscopy (NIRS), near-infrared microscopy (NIRM), near-infrared imaging, liquid chromatography (LC) and olfactometry techniques can and will be applied for the detection of PAPs. NIRS is a non-destructive method that can be applied on-line in feed production plants. Generally, the detection limit is still too high to be applied in official control laboratories. Nevertheless, industrial application is feasible. NIRM and near-infrared imaging are techniques that allow collection of near-infrared spectra from individual particles. The level of detection is lower than 1 g/kg since it is based on the microscopic technique, in combination with the option of identification of the individual particles. LC is based on the detection and, if present, the ratio of different polypeptides. For example, carnosine is mainly present in mammals and anserine mainly found in birds. Olfactometry is based on detection of volatile non-specific agents. It is a non-destructive and fast technique. For both LC and olfactometry it appears that the presence of fish material masks the detection of proteins of land animals, even at a contamination level of 5 g/kg.

Since 2003 five different proficiency studies and ring trials have been organized. The first proficiency study, allowing the participants to apply their own protocol, revealed that correct microscopic detection of 1 g/kg of mammalian PAP in the presence of 50 g fish meal/kg was realised in 0.44 of the cases. However, a bench mark study organized in the same year showed that a microscopic detection of 0.98 can be reached provided the application of an optimal protocol and a sufficient level of expertise. More recent studies showed that training, the application of a decision support system and use of an improved microscopy protocol resulted in a higher sensitivity.

An attractive approach is the combination of the very low detection level of microscopy with identification by other methods. Several strategies for a combination of screening and confirmation methods are discussed in the present paper. The new developments in methodology will support current or new legislation (*e.g.* species-to-species ban, general application of fish meal).

Keywords: BSE; Feed ban; Animal proteins; Microscopy; PCR; Immunoassay; NIR; HPLC; Protein analysis

1. Introduction

In the history of development of feeds with a high nutritional value, materials of animal origin were considered appropriate as ingredients in compound feeds. This inclusion was based on natural feeding patterns of carnivorous (*e.g.* fur animals) or omnivorous (*e.g.* pigs) animals. However, the same strategy appeared to be profitable for herbivorous animals as well. Animal by-products can be readily compared to soy bean hulls, but provide a higher amount of fat as energy source, higher levels of protein and minerals (Ca and P), and supply some essential vitamins. For these reasons formulations including significant

quantities of animal by-products in feeds for cattle, sheep, pigs and poultry had become a common procedure (Grastilleur, 2003; Sellier, 2003). The emergence of bovine spongiform encephalopathy (BSE) as a new disease during the 1990s (Thiry et al., 2004), resulted in an abrupt ending of these practices. It is generally accepted that the most likely route of infection of cattle with BSE is by means of feeds containing low levels of animal proteins (Prince et al., 2003). This view has been widely supported by epidemiological studies, rendering studies and ultimately by the effect of the feed bans (Horn, 2001). Maternal transmission is another transmission route, but it has been estimated that it cannot account for more than about 0.10 of all BSE cases. Furthermore, there is no evidence that maternal transmission occurs in the absence of the feed-borne source (Wilesmith et al., 1997). Because of this likely route of infection, feed bans were enforced, initially for ruminant feeds and later for all feeds for farmed animals.

The first ban prohibited the feeding of mammalian processed animal proteins (PAPs) to ruminants (Directive 94/381/EC). This permanent ban is now enforced in the general TSE Regulation (Regulation 2001/999/EC, EU, 2001). After that, regulations have been introduced for the rendering of animal by-products (1994, amended in, *e.g.* 1995, 1996 and 1999) and for the handling of specified risk material (1997, amended in, *e.g.* 2000 and 2002).

Feeding of by-products to the same species as the source is prohibited (species-to-species ban) by the Animal By-Product Regulation 2002/1774/EC (EU, 2002). Although this is a permanent ban, the enforcement is not active, since the species-to-species ban is temporarily overruled by the extended feed ban. This extended feed ban (Annex IV in Regulation 2003/1234/EC, EU, 2003b) amends the TSE Regulation, in the sense, that all animal proteins from farmed animals are prohibited for feeding to farmed animals again, due to the lack of animal-specific detection methods. After lifting (parts of) this extended ban the species-to-species ban will be actively enforced. Besides the permanent ruminant ban, the feeding of pig material to pigs and avian material to poultry will still be prohibited. Derogations to the feed ban are made, *e.g.* for certain blood products and hydrolysed proteins. Milk and milk products such as whey and egg and egg products are allowed for feeding to farmed animals. There is no legal limit for PAP in feed. This means in other words that a zero tolerance is applied. A more detailed discussion of the feed bans is given by Gizzi et al. (2003a). Recently, a new Regulation has been published (2005/1292/EC—EU, 2005) which provides further derogation for blood products, hydrolysed proteins and for the application of by-products of root and tuber crops processing. In all cases, strict conditions apply.

Feeding of fish material to fish is currently allowed, with the exception of farmed fish material, which cannot be fed to fish of the same species. There is a discussion on lifting the ban on fish meal for ruminant feeds. This would at least solve the problem of fish meal carry-over from pig and poultry feeds that are produced on combined production lines where ruminant feeds are also produced. This proposal requires that PAPs of terrestrial animals can be sufficiently distinguished from fish meal. Whether fish meal is present or absent, in all cases a target detection limit for all methods of 1 g/kg PAP in feed applies. Nevertheless, a sample that contains PAPs below this still infringes the PAP ban.

The current paper will present and discuss the development of techniques for detection and identification of PAP for the support of control measures.

2. Classical microscopy

The screening of samples by means of optical microscopy at several magnifications is currently the only official detection method to enforce the feed ban, capable of detecting meat and bone meal (MBM) as well as other types of PAPs such as feather meal. This official method is documented by Directive 2003/126/EC (EU, 2003a). The key steps are grinding of at least 100 g of sample material through a mesh size of 2 mm, and sieving at least 5 g to obtain a coarse and a fine fraction in which primarily muscle, hair and feather material can be traced if present. Simultaneously at least 5 g of material will be sedimented in tetrachloroethylene (TCE). More dense parts, *e.g.* minerals and eventually bone and fish scale fragments, will sediment at the bottom of the funnel. The sediment as well as the flotation will be dried and sieved to separate coarse and fine fractions (see Gizzi et al., 2003a for a more detailed description). Only minor modifications to the procedure are allowed, according to Directive 2003/126/EC (EU, 2003a). Some laboratories use a conical beaker (Champagne-glass like; Fig. 1, right) for sedimentation, instead of the standard closed sedimentation funnel. This beaker requires a high level of expertise to handle. A specially designed open sedimentation funnel with a half-closed cock at the bottom eases the removal of the sediment (Fig. 1, middle).

Further methodological modifications have been applied in the past and include whether or not to grind or sieve the sample materials, the number and types of solvents, and the embedding agent. Fluids for sedimentation consisting of an additional solvent have been used (French method: addition of tetrabromoethylene (TBE); see Gizzi et al., 2003a) or have been proposed (addition of petroleum ether; Calero et al., 2004). In both cases, two sediments instead of one will be obtained. The objective is to obtain a higher level of "condensation" of the animal fragments, since these fragments are expected to partition into only one of these two sediments. In practice, however, bone fragments might be overlooked if the target sediment is examined only and a higher number of false negative results might appear.

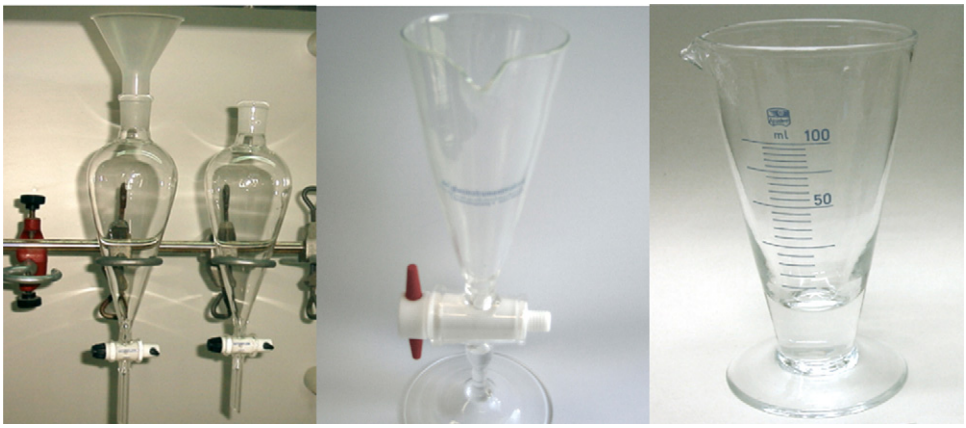

Fig. 1. Several types of glassware used for sedimentation. From left to right: closed separation funnel, open sedimentation funnel, sedimentation beaker.

Results of the latest proficiency test (Boix et al., 2004) with respect to the French method are discussed in Section 7.

Essentially but not exclusively based on bone characteristics, microscopic identification is capable of distinguishing between fish and terrestrial animals, whereas a gradual difference between mammal and avian material has been established (Mondini et al., 1999; Gizzi et al., 2003a; van Raamsdonk et al., 2004). Discriminating characteristics of bone fragments are the shape of the fragments, the shape and density of the osteocyte containing lacunae, the visibility of the canals connecting the lacunae, and the colour and opaqueness of the fragments. These characteristics are extensively documented in the computer program ARIES (2004) (Animal Remains Identification and Evaluation System, Version 1.0, 2004) by means of full descriptions, images and interactive identification trees. ARIES has been developed in the framework of the STRATFEED[1] project and is available for all laboratories. A range of 32 characters pertaining to the lacunae is examined further by using image analysis techniques (Pinotti et al., 2004). The analyses were based on measurements of 30 individual lacunae (13 originating from four mammal samples, 17 from four poultry samples). The major descriptors of the variation between mammal and poultry material are the area polygon (area covered by a single lacuna) and the perimeter (length of the lacuna outline). For 28 lacunae (0.933) a correct identification was made, on two occasions (0.067) the lacunae from poultry bone fragments were incorrectly classified as being mammalian (Pinotti et al., 2004). These findings support the report of Mondini et al. (1999) that certain avian bones show an appearance that is similar to mammalian material, possibly due to growing conditions. The results of Pinotti et al. (2004) indicate that promising prospects exist for optical microscopy.

Besides bone fragments, muscle fibres are major components in MBMs. Currently, the mere presence of muscle fibres is the only parameter that is used concerning muscle material. This is sufficient to support the total ban of PAP for ruminant feeds. Nevertheless, muscle fibres can also be used for discriminating purposes. Parameters for muscle fibre identification have been developed in the framework of the STRATFEED project (van Raamsdonk et al., 2005). These parameters include the width of fibres and the sarcomere length, which are measured from a series of individual fibres. From these measurements, the ratio between these two basic parameters is to be calculated. Typical values for this ratio range from 15 to 60. They are generally lower for poultry than for mammals. Each of these parameters does not show a significant difference between mammal and poultry material, but the set of three parameters allows an indication of the origin to be provided. Especially, the coefficients of variance for these three parameters may indicate the possibility of having a mixture of different MBMs as contamination in feeds. Consequently, the analysis of muscle fibres can support the identification of bone fragments (van Raamsdonk et al., 2006).

Other new developments include the preparation of oil and liquid samples. Proper sedimentation, whether or not preceded by a freeze-drying step, can be applied to these types of samples. Commission Directive 2003/126/EC (EU, 2003a) provides a provisional protocol for oil and fat samples.

[1] Fifth Framework programme European project "Strategies and methods to detect and quantify mammalian tissues in feeding stuffs", abbreviated in the acronym "STRATFEED" and registered under the no. G6RD-2000 CT-00414.

Classical microscopy has its limits for the discrimination at the level of vertebrate classes (mammal *versus* avian). However, terrestrial animals *versus* fish pose no problems. No optically visible discriminating characters are present at lower taxonomic levels (*e.g.* ruminant *versus* non-ruminants). Microscopic detection can easily detect PAPs at lower levels provided a proper application of the method is followed. Detection levels as low as 0.2 g/kg MBM in feed has been reported (Engling et al., 2000). Quantitative detection is part of the official method, but this is optional (Directive 2003/126/EC, EU, 2003a). However, additional screening and confirmation methods are urgently needed for the support of the species-to-species ban to achieve precise identification of the MBMs, such as DNA characterization, protein-based methods or other approaches.

3. DNA detection methods

DNA is a rather robust organic molecule, and the amount remaining after the mandatory heating process of MBM according to EU regulation is generally still sufficient to be able to allow its detection by genetic amplification of taxon-specific animal targets on MBM samples. However, the target sequences for polymerase chain reaction (PCR) have to be sufficient small (*i.e.* smaller than 100 bp) and be present in sufficient amount of copy numbers per cell (Gizzi et al., 2003a). The idea to apply DNA detection methods as an analytical tool for indexing MBM presence in feed was initiated by Tartaglia et al. (1998). Their mitochondrial DNA target is too large for practical purpose in Europe (Myers et al., 2001) even if it appeared to be fit for detection of MBM produced in the United States where the heat treatment is much less severe due to the absence of a wet sterilization step.

Many articles have been published about possible targets for PCR or real-time PCR on detection of MBM in feed since (*e.g.* list reviewed in Mendoza-Romero et al., 2004). However, the DG-SANCO[2] proficiency test of 2003 clearly showed that the state-of-the-art in routine analysis by PCR for the purpose of MBM detection was very poor at that time either with in-house developed methods or with commercial kits (Gizzi et al., 2004). Of all methods used at that time, PCR led to the worst results. Reasons for this disappointing statement were diverse. Most false negative results were probably due to the fact that the degradation of the DNA had been overlooked because of the use of in-house sterilized material and not real-world material coming from rendering plants. The sterilization process conditions applied in those rendering plants appeared to be much more severe than the conditions in laboratory autoclaves. On the other hand, the false positives that were encountered may have resulted from very sensitive PCR techniques in which it appeared to be very hard to have no signal at all even on the negative control samples. The difficulty was to distinguish a significant signal from background noise (Mendoza-Romero et al., 2004).

However, the research efforts of STRATFEED demonstrated that with well-developed protocols and skilful teams, PCR is promising for MBM detection. Especially, real-time (rt-) PCR is of interest due to the use of small targets, certainly if the species-specific ban will come into force. So far it is uncertain if the 1 g/kg contamination level of PAPs can be detected by PCR, since no method has been validated thoroughly for this purpose.

[2] DG SANCO: European Commission General Directorate for Health and Consumer Protection.

Currently, PCR research is aimed to tackle some of its drawbacks. For instance, recent improvements were focused on the size of the test portion of feed in order to improve its representativeness. Up to 10 g (Rensen et al., 2005) was used instead of the 100 or 200 mg usually considered, in combination with the application of an adapted protocol to reduce possible inhibition effects (Sawyer et al., 2005). Optimized DNA extraction methods and proper implementation of the rt-PCR protocol should, therefore, allow detection of adulterations at a 1 g/kg level (*e.g.* Aarts et al., in press; Fumière et al., in press). Heat treatments should not exceed the minimal requirements of the EU regulation considerably. It was shown that an increase of temperature from 133 to 145 °C induced a decrease in target copy numbers of about two orders of magnitude (Chiappini et al., 2005).

A major problem of the DNA method is the fact that it does not distinguish between DNA originating from authorized products (*e.g.* milk and dairy products) and DNA from prohibited materials. An interesting approach to overcome this problem is to work with bone particles from the sediment fraction as in microscopy, since the sediment lacks those authorized ingredients. It is expected that this sample preparation might improve the specificity of the assay (Fumière et al., 2004). Although the sedimentation step eliminates several authorized products, Toyoda et al. (2004) went a step further by washing the sedimented particles with sodium hypochlorite in order to eliminate all possible pieces of authorized material that could stick to bone particles. However, by working exclusively on the sediment fraction, a major advantage of PCR is lost, *i.e.* independence of presence of bone particles. This advantage is of great importance for an appropriate application of the species-to-species ban.

Another promising development is the combination of the NIRM technique with PCR (Fumière et al., 2005). This development takes advantage of both techniques. NIRM can distinguish between a surely authorized animal particle coming, *e.g.* from milk and a possibly prohibited one. PCR applied to this latter single particle can determine its species of origin, if the particle still contains DNA. Thus, it can reveal whether the material has to be considered prohibited material or not within the species-specific ban.

Another PCR application with potential in the control of BSE spread is the ability to separate several methylated forms of a same DNA sequence, which can be tissue-specific. Bisulphite treatment of the DNA will convert the unmethylated cytosine residues into uridine residues, which are equivalent to thymidine residues in PCR. For instance, Gout et al. (2004) used this to detect the presence of brain tissue (which is a specified risk material) in muscle tissue, however still in unheated samples. Further research is required to evaluate the technique for heat-treated MBM. Criteria would be a sufficient sensitivity and signal-to-noise ratio of the methods also at low contamination levels.

The use of DNA-based methods is in some way indirect in this context as in fact proteins should be targeted. Nevertheless, it is mainly because of its potential to differentiate the animal species origin of products that PCR definitely has a future in the range of methods to be used for identification of processed animal proteins.

4. Immunochemistry

A different concept compared to microscopy or polymerase chain reaction (PCR) is based on the analysis of specific proteins in feeding stuffs allowing for the detection of

banned PAPs at various taxonomic levels. The most common approach for the analysis of these proteins is the development of immunoassays, making use of the specific antibody–antigen interaction (Hitchcock and Crimes, 1985; Hofmann, 1997). Though enzyme-linked immunosorbent assay (ELISA) has widespread applications in food and feed analysis, it is important to realise that a positive response regarding the presence of specific PAPs obtained with an immunoassay has mainly an indicative character and needs to be confirmed by other methods such as PCR. This is due to the fact that the target analyte in immunoassay analysis applied to the detection of PAPs is often not sufficiently characterised. Likewise, a complete map of possible cross-reactivities from non-specific proteins is not always available. This is particularly important for the use of immunoassays in this field, given the diverse composition of compound feeding stuffs. Typically, immunoassays are therefore suitable for the screening of a high number of samples for the presence of PAPs. Immunoassays can also be applied in order to *exclude* the presence of *ruminant* PAPs when presence of animal particles is identified by microscopy. Since microscopic analysis is not able to differentiate ruminant bones or from bones of other mammals, a *negative* response of a ruminant-specific immunoassay performed on the same samples would facilitate the risk management of feed ingredients with traces of bones.

Meat identification in food analysis has been applied for several decades (Hitchcock and Crimes, 1985). Ansfield (1994) reported on the development of a ruminant-specific ELISA allowing for the detection of rendered PAPs in feed. This test was also validated in-house by the same group (Ansfield et al., 2000). The test was subjected to a pre-validation trial, since ELISAs have in general the potential of a high sample throughput and because of the claimed specificity regarding ruminant MBMs. This trial was conducted by the European Commission's Joint Research Centre (unpublished results). However, the results indicated that this test was not sensitive enough, especially when the sterilization temperature of MBMs in the feed was above 128 °C. In fact, an important performance criterion for immunoassays to be used in the European Union is their capability to detect traces of MBMs that were sterilized with steam pressure at least at or above 133 °C, 3 bar and 20 min (Regulation EC 1774/2002, EU, 2002).

The availability of correctly sterilized MBMs required for the development and validation of immunoassays is not an easy target, since material from a real-world rendering plant often lacks full characterization in terms of the precise temperature–time profile the material underwent. On the other hand, sterilization conditions of a laboratory autoclave can easier be measured, but material processed in a laboratory autoclave does not always mimic the sterilization conditions as described by European legislation. Various process conditions and their impact on the response of a commercially available ELISA for the detection of beef have been investigated by Pallaroni et al. (2001). This study showed that not only the temperature but also the water content, the particle size and the general set-up of the laboratory experiments are critical factors. The authors showed that these aspects need to be addressed properly to avoid that a test performs well on material prepared in the laboratory but fails when applied to MBMs processed in a rendering plant. Also the required detection limit is very important, since the test should be able to detect at least 1 g PAPs/kg feeding stuff. Here, the detection limit achievable with the European official microscopic method provides a benchmark against which the suitability of alternative methods can be measured. An overview of the characteristics and require-

Table 1
Immunoassays: important characteristics and requirements from European legislation

	Characteristic/requirement	Comment
Target animals	(1) Ruminants; (2a) pigs; (2b) poultry	Highest priority for ruminant-specific test
Target tissue	Sensitivity towards heat stable proteins present in specific animal tissues	Tissue specificity allows for differentiation between authorised and banned proteins but poses problems if not all banned tissues are covered by the test
Design	ELISA or dip stick	Using dip sticks does not require the availability of a fully equipped chemical laboratory (possibility of on-site application)
Limit of detection (LOD)	Required LOD: 1 g PAPs (as mass fraction)/kg compound feed	Required LOD taken from the LOD achievable with the EU official microscopic method. Recent studies confirmed that some of the immunoassays reach this level

ments of immunoassays suitable for the detection of banned PAPs in feed is summarised in Table 1.

More recently various groups both from academic and commercial institutes have developed immunoassays for the detection of MBMs in feed. Chen et al. (2002) published the development of an ELISA based on monoclonal antibodies raised against troponin I. This protein is a regulatory myofibrillar protein isolated from skeletal muscle and shows some striking characteristics. These characteristics are (1) its thermostability as shown in a former study performed by the same authors (Chen and Hsieh, 2000); (2) differences of its molecular structure at various taxonomic levels; (3) its major occurrence in muscle. The latter aspect is important in differentiating between prohibited MBMs and animal proteins that are authorised in animal nutrition. However, when validating the ELISA method the authors did not include feeds adulterated with PAPs at 1 g/kg but exclusively those with concentrations of 10 g and more/kg. Therefore it is uncertain, whether this method would fulfil the criterion set in the European Union regarding the required sensitivity of the analytical methods applied to the detection of MBM in feed (EU, 2003a).

Kim et al. (2004) reported on the development of an ELISA based on a monoclonal antibody raised against h-calderone. This protein is present in all types of smooth muscle but not in skeletal muscle. Interestingly, the response of the immunoassays increased when the temperature and the duration of the heat treatment were raised from 90 to 130 °C and from 10 to 120 min, respectively. The detection limit of the test is claimed to be low (0.5 g/kg), but at that level the signal-to-noise ratio is not sufficient. The results indicate sensitivity towards MBMs from cattle and pigs with signals at very diverse levels. Moreover, cross-reaction was reported for gelatin in general and for feather meal and fish meal (Kim et al., 2004). Therefore, this test cannot be used for identification of material recovered by microscopy because of the lack of specificity.

Two US companies (Strategic Diagnostics – SDI – Inc. and Neogen Corporation) developed lateral flow immunoassays for the detection of MBMs in feed. Lateral flow

immunoassays (dip sticks) offer the striking advantage that they can be utilised on-site (*e.g.* in a feed mill) without complicated laboratory equipment at hand. SDI offers two different dip sticks, which measure total MBMs without differentiation amongst species and mammalian MBMs, respectively. Neogen markets a test especially designed for the detection of ruminant material. These tests have been applied in the laboratory of one of the authors demonstrating their capability of detecting MBM samples that were heat treated at 133 °C and higher (unpublished results). In a recent study (Bellorini et al., 2005), it was shown that the ruminant-specific test mentioned above is able to detect MBM samples treated up to temperatures of 138 °C. The ability to detect MBM traces in fat was also demonstrated.

Immunoassays are expected to play an important role in the detection of PAPs present at trace levels in feeding stuffs. Considering the complex control demands (*e.g.* total MBM ban *versus* species identification) this method has to be integrated in an overall control strategy, combining its specific advantages with those of the other methods.

5. Near-infrared identification

Near-infrared (NIR) spectra of agro-food products are composed of levels of harmonic vibrations related to the fundamental vibration bands found in the mid-infrared region of the electromagnetic spectrum. The origin of the near-infrared bands is the absorption of the near-infrared light by chemical bounds of organic molecules. Spectra of feed ingredients and compound feeds include mainly absorption bands characteristic of the main constituents of the materials; *i.e.* water, proteins, fats and carbohydrates. The traditional main advantages put to the fore of NIR-based methods are rapidity, absence of use of solvents, easiness of application, non-destructive characteristic, reduced costs and the excellent repeatability of the measure. The main limitation of the NIR technique is its indirect nature, *e.g.* it measures no single target such as a specific molecule or proteins. It, therefore, requires measurement of a large range of reference samples for the development of empiric models (Baeten and Dardenne, 2002).

A NIR-based method was first proposed in 1998 for the detection and quantification of MBM in compound feeds (Garrido-Varo and Fernandez, 1998). In this study, a NIR spectrometer and MBM spiked samples at 5–40 g/kg were used. The results showed clearly the potential of the technique to be used for rapid and cost effective detection of animal ingredients in compound feeds. In 2001, the same type of instrument was used to demonstrate the potential of the technique for the detection of terrestrial animal ingredients in fish meal (Murray et al., 2001). More recent studies have confirmed these findings and have stressed the pros and cons of the NIR method for the detection of MBM in compound feed and the discrimination of higher taxonomic groups of species (terrestrial animals *versus* fish; Murray et al., 2004; Pérez-Marin et al., 2004; Garrido-Varo et al., 2005). The main advantage of the method, in addition to the reduced cost and rapidity of the analysis, is the fact that it analyses between 10 and 100 g of the samples allowing to take into account the natural heterogeneity of compound feed samples. This reduces the number of false positives. Presently, the major limitation is the high level of the limit of detection, which still exceeds 10 g/kg and is therefore too high for use in official control laboratories (Gizzi et al., 2003a).

A near-infrared spectrometer coupled to a microscope (NIRM) has also been proposed to tackle the problem of detection of MBM in compound feeds (Piraux and Dardenne, 2000; Baeten et al., 2001, 2005a,b). With a NIR microscope instrument, the infrared beam is focused through a dedicated microscope on each particle of a sample spread on a sample holder and the NIR spectrum is collected. The result of the sample analysis is a successive collection of hundreds of spectra, each one being the molecular near-infrared signature of a particle from one of the feed ingredients used in the formulation of the compound feed. The studies demonstrated the high potential of the NIRM method to detect MBM at a concentration as low as 0.1% in the raw and sediment fraction. The method can also be used to detect fish meal ingredients in the entire sample as well as in the sediment. When using a NIR microscope the subjective judgement of the microscopist is replaced by a particle specific spectrum, which can be subjected to statistical analysis.

The main limitation of the NIRM method is the time required for the collection of particle spectra as it is done in a serial (particle-by-particle) manner. Recent developments in NIR focal plane array (FPA) technology offer a solution to this problem in the form of imaging spectroscopy. It combines the advantages of spectroscopic and microscopic methods along with a much faster sample analysis, since the spectral data are acquired in parallel. An imaging spectrometer gathers spectral and spatial data simultaneously by recording sequential images of a predefined sample; each image plane is collected at a single wavelength band (Baeten and Dardenne, 2002; Bertrand and Baeten, 2005). NIR imaging systems were proposed for the detection of MBM (Baeten and Dardenne, 2002; Fernández Pierna et al., 2004). This technology allows the analysis of about 300–500 particles in 5 min. The simultaneous analysis of hundreds or thousands of spectra using an NIR imaging system has the advantages of the speed and sensitivity that is required for a screening method. The results show that the NIR imaging method has a limit of detection below 1 g/kg for the detection of PAP in the entire sample and in the sediment[3]. NIR imaging and NIRM methods allow the discrimination between most of the fish and terrestrial particles, whatever the fraction (raw or sediment fraction) analysed.

NIR is in principle an interesting approach for being non-destructive and fast but has serious limitations. Notwithstanding the indirect nature of the resulting near-infrared spectra, application at a microscopic level, especially in combined approaches with, *e.g.* PCR identification, is very promising.

6. Other methods

Banned PAPs can be detected in feed samples through more "classical" methods, *e.g.* microscopy, PCR, immunoassays and NIR. However, it is also possible to identify specific animal proteins by high-pressure liquid chromatography (HPLC). Some histidine peptides such as carnosine, anserine and balenine allow for the differentiation between plant and animal proteins. The application to feed analysis has been reported by Schőnherr (2002) and by Concepción Aristoy and Toldrá (2004) presenting a simplified sample preparation

[3] As for classical microscopy, the limit of detection (LOD) of the NIR microscopic method depends on the number of particles analysed.

procedure. Best results have been obtained by using carnosine, which is present at highest concentrations. The studies demonstrated a sufficient relation between the level of adulteration and the calculated area of the resulting curve, which indicates the content measured. Analyses of the individual types of meat revealed that the mass ratio between these peptides differed amongst the various animals (*e.g.* fish *versus* poultry *versus* mammalian). Usually, mammalian proteins contain more carnosine than anserine, whereas more anserine than carnosine is found in poultry material. A higher anserine content has been reported for fish meal as well, although only one fish meal sample has been investigated (Schőnherr, 2002). So, species identification might be possible on the basis of the carnosine/anserine/balenine ratio provided that the contamination includes only one type of PAP (Schőnherr, 2002, 2004). Later, it was demonstrated that the identification of traces of *mammalian* PAPs in the presence of fish meal at high concentration is extremely challenging. In fact, one laboratory applied this method in an interlaboratory study (Gizzi et al., 2004) revealing a very good sensitivity, since all samples containing MBM at 1 g/kg were correctly identified as positive. However, when the samples also contained fish meal at 50 g/kg the specific detection of the MBM traces was not possible.

A new approach is the identification of proteins by mass spectrometry, applying systems with sufficient resolution power such as quadrupole time of flight mass spectrometry (Q-TOF-MS) or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS; Careri et al., 2002). Specific proteins with a unique mass are expected to indicate the species of origin. Large libraries of protein information are provided. More specific targets are peptide fragments of the protein produced by enzymatic digestion (*e.g.*, trypsin). MALDI-TOF-MS has been utilised by Ocaña et al. (2004) in order to detect the presence of gelatin, which was selected as marker substance for prohibited PAPs. Though this approach seems to be suitable to enforce the current total PAP ban in the EU, no indication has been given as to the potential of animal-specific detection.

Olfactometric methods are based on the detection of different volatile compounds in PAPs. Campagnoli et al. (2004) described the detection of non-specific chemical compounds with 10-sensor electronic nose equipment. A set of six samples was used, containing a control (A), one sample adulterated with 50 g/kg of fish (B), two samples adulterated with 5 g MBM/kg (C and D), and two samples adulterated with 5 g MBM/kg as well as 50 g fish meal/kg (E and F). Each of the six samples used was described by its range of 10 output levels and multivariate cluster analysis was used to classify the samples. After cluster analysis a clear difference exists between the control (A) and the other five samples. Furthermore, two clusters appeared, one containing the samples with MBM (C and D) and a second cluster with the samples containing fish meal (B, E and F). In this second cluster no difference was found whether or not MBM was present in the sample (Campagnoli et al., 2004). It has to be concluded that this electronic nose approach is not sufficient for the detection of PAPs of terrestrial animals in the presence of fish meal, even at the relatively high level of adulteration of 5 g/kg. This might be due to the fact that non-specific chemical sensors were used for the detection. Nevertheless, like NIR techniques, the method is non-destructive and could be applied on-line in commercial production plants, *e.g.* to prevent serious carry-over of pure, non-mixed PAPs. Other equipment for measuring volatile compounds, such as direct mass spectrometers (*e.g.* proton transfer reaction–mass spectrometry) may be more sensitive and provide information on particular masses.

7. Ring trials

During the last 3 years, five different intercomparison studies have been organized. The goal was either to perform proficiency testing (test of performance of individual laboratories applying their own method), or to validate the microscopic method (test of the performance of the method). For the first proficiency testing on behalf of DG-SANCO (Gizzi et al., 2003b, 2004) each of the 24 participating laboratories was allowed to apply its own analytical method. As far as microscopy was concerned, the laboratories could also apply the various modifications as foreseen under Directive 98/88/EC (EU, 1998). PCR and immunoassays were also included. The optimized microscopic protocol as developed in the framework of the STRATFEED project was subjected to a validation study (van Raamsdonk and van der Voet, 2003) on behalf of the International Fish meal and Fish oil Organisation (IFFO) with a set of nine experienced laboratories. The microscopic method as described in Directive 2003/126/EC (EU, 2003a) was based on the STRATFEED protocol but slightly modified, *e.g.* for the amount of material to start the sedimentation with (5 g instead of 10 g). This official method was subjected to two studies in 2004 (Boix et al., 2004; von Holst et al., 2005). Further modifications, such as the Austrian method (allowing a *vertical* bottomed sedimentation beaker additional to the basic glass ware, see Fig. 1) and the French approach (using two instead of one solvent for sedimentation), were tested. Recently, the Italian Centro di Referenza Nazionale per la Sorveglianza ed il Controllo degli Alimenti per gli Animali, Aosta (CReAA) organized a proficiency test with a selection of possible treatments. Analysis were requested to be carried out according to Directive 2003/126/EC, with a starting amount of 10 g. Results are distributed exclusively among the participants. In all proficiency tests, the participating laboratories were requested to supply details of the applied method. The basic target in all studies is the detection of 10 g/kg of MBM, whether or not in the presence of 50 g fish meal/kg. Samples with 5 and 50 g feather meal/kg as an alternative representation of PAPs were added in the DG-SANCO study of 2004 (Boix et al., 2004).

In the first DG SANCO proficiency test from 2003 the laboratories applied methods that only complied with the former protocol of Directive 98/88/EC (EU, 1998). However, this study is included in this overview to demonstrate the improvement of the microscopic method gained in the last years.

The microscopic results are presented in Table 2. The following conclusions can be drawn from the various studies:

- The resulting specificity is generally high in most cases. In certain studies, however, a surprisingly high number of false positive results was reported. For instance, proportions of 0.71 and 0.81 for the incorrect indication of the presence of MBMs in general and of land animal material, respectively, were found after applying the Austrian method. This means that a false positive result for terrestrial animals was reported for 0.19 of the blank samples and additionally 0.10 erroneous indication of the presence of fish material. Specificity appeared to be at acceptable levels in both the IFFO and the CReAA studies. Both studies were based on selected sets of nine laboratories.
- For the material containing 1 g MBM/kg in the presence of fish meal the values of the sensitivity obtained in the STRATFEED validation study and the DG SANCO study 2004

Table 2
Results of intercomparison studies for detection of animal proteins expressed in specificity (proportion of correct negative results: blank samples) and sensitivity (percentage of correct positive results) within the seven (sub-)studies

(Sub-)study and tested microscopic protocol	Specificity			Sensitivity		Amount of starting material (g)
	Blank[a] (all animal proteins)	Blank[a] (land animals)	50 g fish meal/kg feed[a] (land animals)	1 g MMBM/kg feed[a] (land animals)	1 g MMBM/kg feed with 50 g fish meal/kg[a] (land animals)	
DG-SANCO (Gizzi et al., 2003b, 2004): 98/88/EC	0.94	0.94	1.00	0.92	0.44	Free
IFFO (van Raamsdonk and van der Voet, 2003): Stratfeed	1.00	1.00	0.94	1.00	0.987	10
Stratfeed (von Holst et al., 2005): 2003/126/EC	0.89	0.91	0.86	0.99	0.77	5 or more
DG-SANCO (Boix et al., 2004)						
Austrian method: 2003/126/EC, modified	0.71	0.81	1.00	0.95	0.76	5 or 10
New member states: 2003/126/EC	0.78	0.91	0.84	1.00	0.66	5 or 10
French method: 98/88/EC, modified	0.86	0.93	0.86	0.93	0.60	10
CreAA (2005): 2003/126/EC	0.95	0.98	1.00	0.944	Not included	10

A rare presence of only one bone fragment was not reported as a positive, but assumed to be due to lab contamination. The Stratfeed protocol was used a basis for Directive 2003/126/EC (difference is the amount of starting material), the French method is a modification of 98/88/EC, the Austrian method is a modification of 2003/126/EC. The amount of sample material used for sedimentation is indicated in the rightmost column.

[a] Tested material.

(Austrian method) were almost equivalent (about 0.77). The high sensitivity (0.987 in the presence of fish meal) from the IFFO study reflects most likely the high expertise of the involved laboratories.

- Feather meal was detected in the samples contaminated at 5 and 50 g/kg with a sensitivity of 0.808 and 0.986, respectively (Boix et al., 2004).
- Comparing the results from the DG SANCO study 2003 and the STRATFEED validation study it may be concluded that the improved protocol of Directive 2003/126/EC (EU, 2003a) is significantly better than the one established by former Directive 98/88/EC (von Holst et al., 2005).
- Three laboratories participating in the DG SANCO 2003 *and* the STRATFEED validation study obtained an overall accuracy for all materials of 100% and identified MBM in all samples containing 1 g MBM/kg in the presence of fish meal. Two other laboratories obtained slightly inferior results. These results emphasise again the importance of the necessary expertise of the laboratory.
- Given the proven potential of the improved microscopic method, the continuous organisation of training courses, workshops and proficiency testing will further improve the ability of European laboratories to detect PAP at 1 g/kg in the presence of fish meal.
- Another important improvement is the development of a PC-based expert system "ARIES" developed in the STRATFEED project that assists the analyst in identifying banned PAPs.

Based on the supplied data on methodology details by all participants, there appears to be a positive relation ($R^2 = 0.564$) between the specificity and the amount of sample material used for sedimentation. At least a modification of the current official method to require a starting amount of 10 g of material for sedimentation would lead to further improvement of the performance of the method.

DNA detection by means of PCR was included in the DG-SANCO study (Gizzi et al., 2003b, 2004). Although probably not that convincing at that time, PCR as technique has certainly a place next to other techniques like microscopy or immunochemistry in the control of presence of MBMs in feed (see Section 3). Recent in-house validation tests demonstrated promising results (Chiappini et al., 2005; Aarts et al., in press; Fumière et al., in press). Even if it will not solve all problems because it has its own limits, the technique has a real potential certainly if species identification becomes an important issue. Concerning routine application of the technique, this should not be a problem as throughout Europe many laboratories are equipped to detect GMOs in a quantitative manner, with identical technical requirements.

Immunoassay methods were included in two of the five studies. The DG-SANCO study (Gizzi et al., 2003b, 2004) revealed that for the parameter "*mammalian* PAPs" the detection of feed adulterated with 1 g/kg MBM was extremely challenging for all the immunoassays included in the study, which was indicated by a high number of false negative results. However, at an MBM concentration of 5 g/kg no false negative results were reported. Another problem was a lack of specificity observed for the samples containing fish meal but no MBM as shown by an increased number of false positive samples. Unfortunately, this study did not allow for a full evaluation for the parameter "*ruminant* PAP", since the MBM utilised in this study contained only 500 g bovine material/kg, thereby leading to a true concentration

of *ruminant* PAPs in the feed samples of 0.5 and 2.5 g/kg. In fact, many samples containing the ruminant MBMs at the lower concentration level were reported as false negative. In the second DG-SANCO study (Boix et al., 2004) the most promising tests from the former study were subjected to a pre-validation, which focused on the detection of ruminant PAPs. The laboratories applied their own method on the same set of test samples. Again, 1 and 5 g MBMs/kg compound feed were the target concentrations, but this time the MBMs utilised were pure ruminant material. Four companies participated in the study, but one laboratory did not report results, another laboratory applied two methods and two laboratories utilised one method, respectively. The results clearly demonstrated that the heat treatment of the test material did not pose a problem, since the fraction of correct positive results was independent of the applied temperature (139 and 133 °C). Two methods were able to detect MBM at a concentration as low as 1 g/kg and deserve further evaluation, especially in terms of specificity on different feed ingredients. The methods were not subjected to samples adulterated with feather meal, since specificity was not claimed to this special type of PAP. It can be concluded that the performance profile of immunoassays developed for the detection of banned PAPs in feed has been significantly improved in the last years in terms of sensitivity and specificity. Therefore, it can be expected that immunoassay will play an important role in the future control strategy of feeding stuffs regarding the enforcement of animal-specific ban of PAPs.

8. Discussion

In the sections above dedicated to the various methods, it was evident that the accuracy of methods has improved significantly within the last years (*e.g.* Table 2 and references therein; Baeten et al., 2005c). Future use of classical microscopy should focus on the organisation of training courses and proficiency tests. The current European protocol should be improved with regard to some technical details such as a fixed starting amount of 10 g instead of the currently set minimum of 5 g, as suggested by the results of van Raamsdonk and van der Voet (2003).

PCR, in principle, could be the future cornerstone of feed analysis, since it combines both requirements of a high species specificity and high forensic value of the result. Several research developments (Chiappini et al., 2005; Aarts et al., in press; Fumière et al., in press) resulted in a significant improvement of a specific PCR protocol that still needs to be confirmed in an intercomparison study.

Significant improvements were also gained in immunoassay technique (*e.g.* Bellorini et al., 2005) and there is no doubt that some approaches are ready for final validation studies. Given the simplicity of these tests, their high sample throughput, the proven sensitivity and selectivity, immunoassays will play an extremely important role in the future feed safety strategy.

NIR-microscopy and the more advanced instruments such as IR camera offer non-destructive methods that have shown their potential in feed analysis with the possibility of handling high numbers of samples with proven performance characteristics (Baeten et al., 2005b). However, this approach still needs to be thoroughly validated, before application by other laboratories can be considered.

HPLC of histidine di-peptides and olfactometry methods are both based on very general animal or even non-specific markers. Although a sufficient level of detection might be reached, the detection of PAPs from terrestrial animals in the presence of fish material is virtually impossible due to the non-specificity of the presently selected markers.

Detection of PAPs by means of the official detection method, *e.g.* classical microscopy, has been pinpointed for weaknesses such as duration of the analysis, need of skilled staff, differentiation power limited to higher animal taxons and inaccurate quantification of animal material. The use of toxic solvents has been stated as well (Gizzi et al., 2003a). The strengths of classical microscopy, however, are its high sensitivity even at low contamination levels as confirmed in several validation studies and its independence with respect to heat treatments to which MBMs were subjected. The throughput of classical microscopy can be compared to several other methods. The weakness of a minimum required level of expertise was recognised and solutions have now been developed in the form of expert systems such as ARIES.

There is a need for applying separate screening and confirmation methods and several strategies for combining these methods have been proposed (Baeten et al., 2005c). A possible scenario is to test feed samples by using immunoassay methods in the form of dip sticks, followed by confirmation using classical microscopy. Such a scenario combines the benefit of a short application time of dip sticks with the high reliability of microscopic detection, but the number of false negatives at lower contamination levels (Gizzi et al., 2004) is a disadvantage of this combination. On the other hand, Boix et al. (2004) reported a significant improvement of the sensitivity of immunosassays indicating the suitability of some of the methods evaluated, which, however, still need to pass validation. Another approach is microscopy as screening method for the almost absence of false negatives and the currently available knowledge, with either immunoassay or PCR as confirmation methods. rt-PCR can be applied to the sediment resulting from the microscopy protocol (Fumière et al., 2004; Toyoda et al., 2004). In this way, the problem of false negatives after applying immunoassay and PCR is avoided, since only positive samples need to be confirmed, and both methods are able to indicate the source of the animal proteins in order to support the species-to-species ban. Other approaches are screening by NIRM techniques followed by PCR or immunoassay confirmation (Fumière et al., 2005) or combination of classical microscopy and either PCR or immunoassay. A very firm way to prove the presence of prohibited materials is mass spectroscopy, *e.g.* Q-TOF or MALDI-TOF, as confirmation method.

The European Commission has recently published a plan for future developments in legislation. With respect to the feed ban a wider application of fish meal, a higher tolerance than zero content for solving the problem of unavoidable environmental contamination with bone fragments (*e.g.* in beet pulp), a wider application of tallow, and lifting the general ban to get the species-to-species ban for non-ruminants in effect, are being discussed. Some of these relaxations have to be based on quantitative risk assessments.

The current total ban is expected to be replaced by a species-to-species ban and more diversification would be required, including quantitative measures. The new developments for screening and confirmation methods for animal proteins in feeds indicate that a proper and adequate support of legislation can be foreseen, including those new derogations.

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