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

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

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

6.1 Introduction

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

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

6.2 Legislative framework and requirements

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

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

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

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

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

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

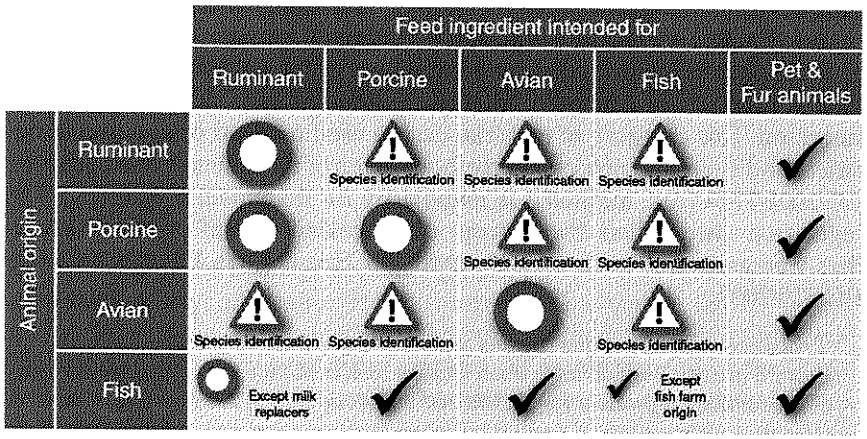

Fig. 6.1 Overview of the current feed ban in Europe. Circle signs refer to permanent prohibition; triangular warning signs refer to future possibilities of lifting the prohibition provided species identification is possible. Tick marks refer to authorised use.

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

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

6.3 Future legislative trends in the EU

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

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

6.4 Detection and identification of processed animal proteins

6.4.1 Official method

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

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

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

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

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

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

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

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

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

Even if the official method for qualitative analysis offers sufficient reliability as it stands, at least for frontline purposes, results from past studies also stressed the need for further fine tuning of the method. Actually too many aspects of the method are subject to interpretation because of a lack of precision. Claims that deviations between qualitative results are linked to these aspects are regularly made (e.g., Veys and Baeten, 2007a; van Raamsdonk *et al.*, 2008). For instance, the initial portion of at least 5 g of sample material to be used for preparing the different fractions should be fixed at a higher value. Although from past tests there is no statistically significant evidence of any correlation between the amount of the initial portion used for the sedimentation and sensitivity scores, it is suggested that a fixed value of 10 g should be used in the future. Apart from statistical theoretical models and reflections (Murray *et al.*, 2005; van Raamsdonk *et al.*, 2008), the reason for this amount is clear: the more material used, the more sediment can be recovered. This is crucial, especially when contamination levels below 0.1% PAPs have to be detected, indicating scarce animal particles in the feed: more sediment leads to a higher probability of presence of animal particles. The diversity of vials for recovering sediment could also be a source of heterogeneity (van Raamsdonk and van der Voet, 2003), at least with regard to the reproducibility of qualitative analysis because of quality variations in the obtained sediment, such as the relative concentrations of minerals vs. bone content. This could also partly be a source of variation in quantitative analyses because the type of vial could account for variations in recovered sediment weights (van Raamsdonk *et al.*, 2008). Gizzi *et al.* (2003) stressed the need for harmonised slide preparation for obtaining comparable results among laboratories, but this remains unchanged. On the use of mounting media, the official protocol does not specifically refer to any measurable viscosity unit that would be required for glycerol and for the most variable chemicals such as commercial paraffin oils. Embedding media that are too fluid, for instance, should be avoided because due to rapid filling of lacunae, the number of bone particles could be overlooked. Recently the use of Norland Optical Adhesive 65 for permanent slide preparation has been proposed by Veys and Baeten (2010). This resin preserves all the optical properties needed for distinguishing bone fragments, while offering long-term conservation of slides. The number of slides that need to be observed is also discussed because of unclear instructions in the different cases. Instructions on the type of slide – hollow or classical, size of coverglass, amount of material to spread on a slide, density of the slide (number of particles over unit area) – are also lacking. Fixing similar parameters for the use of the microscopic method is needed because it is still dependent on versatile parameters – the analyst's skills and human observation capabilities – which are both subjective.

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

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

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

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

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

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

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

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

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

6.4.2 Alternative methods

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

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

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

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

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

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

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

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

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

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

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

6.5 Detection and quantification of glyceroltriheptanoate (GTH)

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

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

6.6 Future analytical methods

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

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

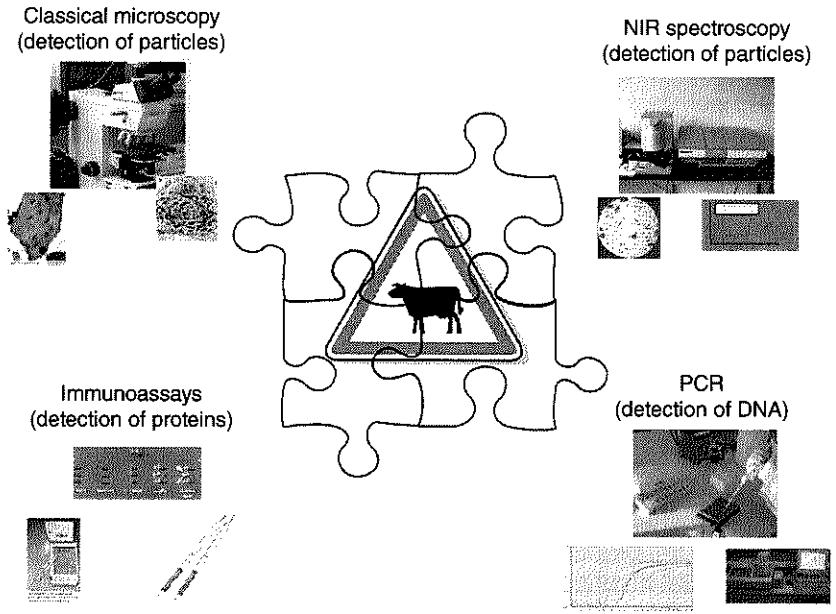

Fig. 6.2 Identification of prohibited animal by-products in feed is like a jigsaw. A final realistic vision on the type of adulteration can only be achieved by applying a multiple method approach.

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

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

6.7 Conclusion

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

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

6.8 Sources of further information and advice

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

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