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An Overview of the Legislation and Light Microscopy for Detection of Processed Animal Proteins in Feeds

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ABSTRACT From the first cases of bovine spongiform encephalopathy (BSE) among cattle in the United Kingdom in 1986, the route of infection of BSE is generally believed by means of feeds containing low level of processed animal proteins (PAPs). Therefore, many feed bans and alternative and complementary techniques were resulted for the BSE safeguards in the world. Now the feed bans are expected to develop into a "species to species" ban, which requires the corresponding species-specific identification methods. Currently, banned PAPs can be detected by various methods as light microscopy, polymerase chain reaction, enzyme-linked immunosorbent assay, near infrared spectroscopy, and near infrared microscopy. Light microscopy as described in the recent Commission Regulation EC/152/2009 is the only official method for the detection and characterization of PAPs in feed in the European Union. It is able to detect the presence of constituents of animal origin in feed at the level of 1 g/kg with hardly any false negative. Nevertheless, light microscopy has the limitation of lack of species specificity. This article presents a review of legislations on the use of PAPs in feedstuff, the detection details of animal proteins by light microscopy, and also presents and discusses the analysis procedure and expected development of the technique. *Microsc. Res. Tech. 74:735–743, 2011.*

INTRODUCTION

Bovine spongiform encephalopathy (BSE) commonly known as "mad cow" disease is a chronic, degenerative disorder affecting the central nervous system of cattle. The occurrence of BSE and its transmissibility to humans is quite serious also due to the fact that it poses a public health threat. Strong epidemiologic and laboratory evidence indicates that a new variant of Creutzfeldt-Jakob disease (vCJD) caused by BSE may be transmitted to humans by consumption of contaminated products with BSE agent (Bruce et al., 1997; Scott et al., 1999). Unfortunately, the diseases are invariably fatal for humans and there is no known treatment or cure.

The first case of BSE among cattle was described in the United Kingdom (U.K.) in 1986, and then in the European Union (EU) the identification of cases of BSE was led to Belgium, Denmark, France, Ireland, Liechtenstein, the Netherlands, Portugal, Switzerland, Germany, Spain, and Italy. In December 1993, one BSE case was diagnosed in Canada. Later it was confirmed that the infectious cattle was imported from U.K. in 1987. This epidemic spread worldwide quickly; the first cases of BSE in Asia were reported by the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) in 2001. In 2003, a dairy cow has been diagnosed to be infected with BSE in America. It was promulgated in 2007 by the Organization Internationale des Epizooties (OIE) that there were about 25 countries involved in the infection of cattle with BSE. From 1989 through June 2007, more than 190,000 cases of BSE had been reported in these countries (http://www.oie.int/).

It is generally believed that the most likely route of infection of BSE is by means of feeds containing low level of animal proteins (Prince et al., 2003), and the consumption of infected material by ruminants is recognized as the main BSE transmission pathway (European Commission, 1998). Epidemiological evidence established that the outbreak of BSE was related to the production and use over many years of meat-and-bone meal contaminated with BSE agent, and it was amplified by feeding rendered bovine meat-and-bone meal to young calves. This likely route of infection of BSE resulted in many feed bans in the world and also some detection methods. Now the most severe feed ban is carried on in the EU, the processed animal proteins (PAPs) of mammalian and avian origin are prohibited in all of the animal feed except pet.

Currently, banned PAPs can be detected by various methods as light microscopy, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), near infrared spectroscopy (NIRS), and near infrared microscopy (NIRM). However, light microscopy is the only accepted and the most reliable method for enforcing the current total feed ban in the EU comparing to the other methods (Fumière et al., 2009; Gizzi et al., 2004).

This article presents a review of legislations on use of PAPs in feedstuff and the detection details of animal proteins in feedstuffs by light microscopy. It also

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TABLE 1. Principal legislations for feeding of animal specific proteins in the EU

Materials	Livestock				
	Ruminant	Pig	Poultry	Fish	Pet animals and fur
Ruminant	Permanently prohibited (EC/999/2001)	Temporary prohibited (EC/1234/2003)	Temporary prohibited (EC/1234/2003)	Temporary prohibited (EC/1234/2003)	Permitted
Pig	Permanently prohibited (EC/999/2001)	Permanently prohibited (EC/1774/2002)	Temporary prohibited (EC/1234/2003)	Temporary prohibited (EC/1234/2003)	Permitted
Poultry	Temporary prohibited (EC/1234/2003)	Temporary prohibited (EC/1234/2003)	Permanently prohibited (EC/1774/2002)	Temporary prohibited (EC/1234/2003)	Permitted
Fish	Temporary prohibited except for milk replacer of young ruminants (EC/956/2008)	Permitted	Permitted	Permanently prohibited for farmed fish (EC/1774/2002)	Permitted

presents and discusses the procedure and expected development of the technique.

LEGISLATIONS ON USE OF PAPs IN FEEDSTUFF

In 1996, the World Health Organization (WHO), OIE and Food and Agricultural Organization (FAO) agreed five recommendations for measures against BSE. The details were as follows: (1) no part or product of any animal which has shown signs of transmissible spongiform encephalopathies (TSE), nor tissues that are likely to contain BSE agent should enter into the food chain (human and animal); (2) all countries should establish surveillance and compulsory notification of BSE cases; (3) all countries should ban the use of ruminant tissues in ruminant feed; (4) milk and tallow are considered as safe; (5) gelatin and tallow are only considered as safe provided effective rendering procedures are used (WHO, 1996).

The first ban of BSE in the EU was regulated by Directive EC/381/94, which prohibited the use of mammalian PAPs in ruminant feed. This was enforced in the Regulation EC/999/2001. After that a species to species ban was amended by Regulation EC/1774/2002 prohibiting feeding of animals with processed proteins from the same species. Later on Regulation EC/1234/2003 extended the ban: all animal proteins from farmed animals were prohibited for feeding farmed animals again. In 2005, another regulation was published in the EU (EC/1292/2005), which provided further derogation for blood products, hydrolyzed proteins and for the application of by-products of roots and tuber crops processing. It is stipulated recently in Regulation EC/956/2008 that the use of fish meal is allowed for young animals of ruminant species which is limited for the production of milk replacers. According to the EC legislations on BSE regarding animal feed, situations of animal specific proteins is summarized in Table 1.

It is now hypothesized that BSE entered North America during the1980s when Canada and the U.S. imported a limited number of cattle from the U.K. BSE then has been a reportable disease in Canada and the U.S. In 1990, Canada banned the importation of cattle from the United Kingdom and Republic of Ireland, and then beef products from European countries not free of BSE were also officially banned. The U.S. also introduced similar import measures. Canadian and American feed restrictions are virtually identical. Although there are some small differences (for example, Canada prohibits the feeding of poultry litter and plate waste to ruminants, whereas the U.S. does not). Both Canada and the U.S. perform regular inspections of industry to verify compliance with their feed ban requirements. In addition, the tissues, known as specified risk material (SRM), are removed from all animals slaughtered for human consumption to maintain food safety in the U.S and Canada, and SRMs are defined as: skull, brain, trigeminal ganglia, eyes, spinal cord, distal ileum, and the dorsal root ganglia of cattle aged 30 months or older. While in the United Kingdom and other countries classified as moderate to high risk, the SRMs include tonsils and intestines in cattle at all ages; brains, eyes, spinal cord, skull, and vertebral column form animals over 12 months of age. In the EU, SRMs are excluded by law from the human and animal food chain (http://healthymeals.nal.usda.gov/fsrio/document_fsheet.php?product_id=169). In 2004, the use of encephalon and the other tissues derived from cattle with the risk for BSE was prohibited in food and cosmetics processing in the U.S.

In Middle East and Asia, only Israel and Japan have been involved in the infection of cattle with BSE according to OIE reported in February 29, 2008 (http://www.oie.int/). Various measures have been taken in Japan to protect the cattle population from exposure to feed potentially contaminated with the BSE agent (Table 2). The MAFF introduced a "complete feed ban," which prohibited the use of all animal protein (including mammal, poultry and fish protein but excluding milk protein) in feed for ruminants, swine and poultry just after the detection of the first case of BSE in Japan in 2001. Later this feed ban was altered to allow the use of proteins derived from swine and poultry in feed for swine and/or poultry on the condition that these proteins are produced in dedicated plants where no ruminant materials are handled (Shinoda et al., 2008). The food industry wastes including proteins of mammal, poultry, fish, and shellfishes are prohibited to fish currently.

Although there are no BSE cases in China by now, many regulations and measures have been applied to ensure the safety of the feed chain. China banned the importation of PAPs from the countries with BSE cases. It is stated by the Ministry of Agriculture of China in 2001 that PAPs are prohibited for feeding of ruminants (Chinese Regulation, 2001). Another regulation was published in 2004, which provide the rules for management of feed products of animal origin (Chinese Regulation, 2004). All the feed products of animal

TABLE 2. Principal legislations for feeding of animal specific proteins in Japan

Materials	Origin of materials	Livestock			
		Ruminant	Swine	Poultry	Fish
Gelatin, collagen	Mammal	0	0	0	0
Milk, dairy products					
Powdered blood, plasma	Ruminant	x	x	x	x
	Swine, horse, poultry	x	0	0	0
Fish meal	Fish and shellfishes	x	0	0	0
Chicken meal, feather meal	Poultry	x	0	0	0
Hydrolysis protein, steamed bone meal					
Meat and bone meal,	Swine	x	0	0	0
hydrolysis protein, steamed	Swine-poultry mixture	x	0	0	0
bone meal	Ruminant	x	x	x	x
Food industry wastes	Mammal, poultry,	x	0	0	x
including animal protein	fish and shellfishes				

0 = authorized; x = prohibited.

origin and manufactories should be registered, and all the production lines (workshop, establishment, technics, detection of product quality and environment, etc.) should strictly follow the rules constituted by the Ministry of Agriculture. For the feed products quality of animal origin, national standards are amended for fish meal (GB/T 19164-2003) and bone meal, meat and bone meal for feedstuffs (GB/T 20193-2006) respectively. In 2002, the use of imported materials derived from cattle was prohibited in human drugs by the State Food and Drug Administration of China. The BSE institute (Conducting research on BSE) and reference laboratory (Conducting detection on BSE) was founded in 1998 and 2001 respectively in China. There is also a central organization in China which belongs to the Ministry of Agriculture performs regular inspections of feed industry to ensure the application of regulations.

The application of the regulations calls for accurate, precise, and reliable methods to be at the legislator's disposal. Now various methods have been developed to identify PAPs in feedstuffs. In the U.S., Canada, and the EU, light microscopic evaluation is currently the only standard analytical method for the determination of constituents of animal origin for the official control of feedstuffs. The light microscopy with ELISA and PCR method comprehensively are the MBM detection methods authorized by MAFF departmental regulations in Japan. In China, PCR technique is the only official control method to detect constitutes of ruminant in feed. Now the feed bans are expected to develop into a "species to species" ban, which prohibits only the feeding of animal specific proteins to the same species (The TSE Roadmap, 2005). Therefore, the species-specific identification methods would be required in the future.

LIGHT MICROSCOPY FOR DETECTION OF PAPs IN FEEDSTUFF

The light microscopy method is based on the analysis of remains of tissues for the detection of PAPs in feed. It includes both the observation of morphological conformations of rough fragments with a stereomicroscope and the examination of histological structures of fine particles with a light microscope (AOAC, 1998). The guidelines for the identification of constituents of animal origin by microscopy were first given by European Commission Directive EC/88/1998 to enforce the feed ban, and later this former official method was modified by EC/2003/126. Recently, the EC/2003/126 text was replaced by Annex VI of Commission regulation EC/152/2009. The light microscopy is the only validated and accepted official method in EU to detect the presence of animal proteins. Other methods can be applied in EU but in support to the microscopic one. Microscopic analysis can detect animal proteins in the form of meat and bone meal (MBM) at sufficiently low levels (<0.1%), with hardly any false negative (Engling, 2000). Latest proficiency tests organized for Animal Protein detection demonstrated that the method is very efficient but requiring continuous advancement and training of the operators (van Raamsdonk et al., 2008; Veys and Baeten, 2007b).

Sample Preparation for PAPs Detection by Microscopy

Figure 1 shows the flow diagram of the analysis procedure for microscopic detection according to Commission regulation EC/152/2009 and STRATFEED (one of the FP5-funded projects which aimed at developing new technologies for the detection and quantification of illegal addition of mammalian tissues in feedstuffs) (van Raamsdonk et al., 2005). The key steps are grinding of at least 50 g of sample materials to pass a 2-mm mesh sieve, and sieving at least 5 g of the ground sample with a mesh size of 0.5 mm to obtain a coarse and a fine fraction. If the feed sample is contaminated with MBM, both fractions may contain bone particles, muscle fibers, hairs or feather filaments and egg shells or fish scales besides the usual vegetal feed ingredients. Simultaneously, at least 5 g of ground sample will be sedimented in a separation funnel with tetrachloroethylene (density 1.62). More dense parts, i.e., minerals and eventually bone and fish scale fragments, will settle down at the bottom of the funnel. The sediment as well as the flotation will be dried and sieved (0.5 mm) to separate coarse and fine fractions. The advantages of the sedimentation procedure are the concentration and selection of bone particles from the feed, and the de-fatting of the material in the tetrachloroethylene, which gives a clearer view of the particles. The coarse fractions are examined by stereomicroscope with reflected light directly, and the fine fractions are observed by compound microscope with transmitted light or polarized light. Slides for the compound

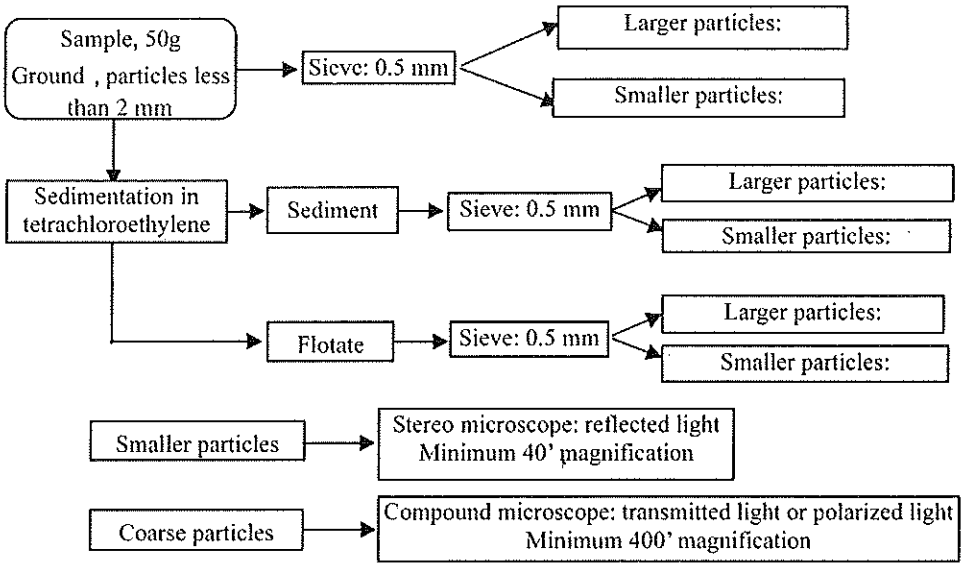

Fig. 1. Analysis procedure for microscopic detection.

microscope at higher magnifications are made principally in glycerol or paraffin oil, and some reagents might be used for specific stainings: Alizarin Red (staining of bones), Fehling reagent (detection of muscle), and cystine reagent (detection of hairs and feather filaments).

There are some modifications of this procedure applied in different laboratories, such as the use of conical beakers like champagne-glass instead of separation funnels for the sedimentation (so called Austrian method, Boix et al., 2004). To remove the sediment easily, a specially designed open sedimentation funnel with a half-closed cock are sometimes used. Liquids for sedimentation consisting of an addition of tetrabromoethylene (so called "French method," Michard and Ziebal, 1999) or addition of petroleum ether (Calero et al., 2004) were proposed in the past. In both cases, two sediments instead of one would be obtained with the intention to gain a higher level of "condensation" of the animal fragments. The microscopic method as described in Directive EC/2003/126 was based on the STRATFEED protocol but modified the amount of material for starting the sedimentation with 5 g instead of 10 g, this is also focus for discussion. In order to perform proficiency testing or to validate the different microscopic methods, different interlaboratory studies have been organized (Table 3). Studies were organized by the International Fish meal and Fish oil Organization (IFFO) and the Italian Centro di Referenza Nazionale per la Sorveglianza ed il Controllo degli Alimenti per gli Animali (CReAA) according to Directive EC/2003/126, with a starting amount of 10 g. To study whether Austrian method and French method would gain comparable results to the current standard method, an intercomparsion study had been conducted on behalf of European Commission's Directorate General for Health and consumer protection (SANCO) in 2004. Results showed that specificity was at acceptable level in both the IFFO and CReAA studies. A high number of false positive results were found after applying the Austrian method. It is indicated that for most of the materials, results of the French method are comparable with the results of the Directive EC/2003/126. The discussions and improvements of different methods are still on going now.

Qualitative Analysis of PAPs by Light Microscopy

The qualitative analysis of PAPs by microscopy relies on the morphological characteristics of the different animal particles which are found back from the different fractions obtained (cf. previous section). The main category of particles of animal origin that are present in feeds are bones and muscle fibers. Additional particles such as cartilage, hairs, feather filaments, egg shells, fish scales, and ligaments may also be present. Parts from organs, skin, and other soft tissues are generally absent or if present barely detectable. Of these particles, bones appear to be the most persistent, even with the current EU rendering practice of sterilization at 133°C and 3 bars for 20 min (van Raamsdonk et al., 2005).

The typical long bones show a pattern of lacunae containing the bone cells, usually organized around a central canal, or Haversian canal, in sets of circular lamellae. The lacunae are connected to each other and to the central canal by very fine network of canaliculi. In cartilage, the cells form holes that are more globular and there are no canaliculi. General descriptions for major vertebrate classes (mammal, avian, and fish) can be provided but there is a large variation (Gizzi et al., 2003; van Raamsdonk et al., 2005). Bone particles from mammals (ruminants and porcine) are of the same shape, both at higher magnifications show a more or less globular appearance with elliptical to almost globular lacunae (Figs. 2 and 3). Canaliculi may be visible, depending on the quality and opaqueness of the bone particle. The orientation in circular lamellae is sometimes visible on larger bone fragments. The bone particles from poultry usually show a more splintered (sharply edged) appearance under the stereomicroscope, caused by the different structure of the

TABLE 3. 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)

(Sub-)study and tested microscopic protocol	Specificity			Sensitivity		
	Blank[a] (all animal proteins)	Blank[a] (terrestrial animals)	50 g fish meal/kg feed[a] (terrestrial animals)	1 g MMBM/kg feed[a] (terrestrial animals)	1 g MMBM/kg feed with 50 g fish meal/kg[a] (terrestrial animals)	Amount of starting material (g)
IFFO (van Raamsdonk and van der Voet, 2003): STRATFEED	1.00	1.00	0.94	1.00	0.987	10
CreAA (2005): 2003/126/EC	0.95	0.98	1.00	0.944	Not included	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 (von Holst et al., 2006): 98/88/EC, modified	0.86	0.93	0.86	0.93	0.60	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. Sources: (van Raamsdonk et al., 2007).

air-filled bones. Lacunae are more globular and their density is higher than in mammal bone particles. Canaliculi are generally not visible. Fish bones show a completely different pattern, they are often parallel sided and tube shaped (Figs. 2 and 3). Lacunae in fish bones of herring and related species are usually elongated, with a clear fusiform network of irradiating canaliculi. A different structure is found in cod and related species, where the lacunae are linear without visible canaliculi. It is proved by the facts that staining does not affect the features of the bone fragments, moreover a better detection could be realized by Alizarin Red staining on bones. The cartilage structures of mammals, poultry and fish show mostly globular lacunae without connecting canaliculi (Fig. 3).

Notwithstanding this appearance of cartilage, fish meal is usually distinguishable from the slaughter byproducts of terrestrial animals because of the typical shape and appearance of the lacunae and canaliculae but also as other structures such as fish scales, and sometimes otolith, are present. The difference between poultry and mammal meal is more difficult to detect and there are overlaps in the range of characteristics. The EU legislation does not make a distinction between different terrestrial animals and therefore only the presence of bone particles is currently sufficient to reach a positive conclusion. Both smooth and striated muscles are present in meal derived from mammals, poultry, and fish. They are basically used to state the presence of animal proteins in general. Muscle tissue is present as single fibers, which are broken into relatively short fragments (Fig. 3). The width of the fibers depends largely on the state of nutrition of the animal and the treatment during slaughter (Devine et al., 2002). Additional types of particles such as hairs, feather filaments, eggshells, fish scales, and fish gills may help to confirm the presence of animal meals in feed. Hairs will point to mammals as the source, feather filaments and eggshells to avian material, and fish scales to fish. Nevertheless, the presence of one of these fragments does not exclude the possibility of a mixture from more than one animal origin. Although most rendering plants now have separate production lines for the different classes of vertebrates, carry-over or unintended contamination with rodents or other animals cannot be excluded (Gizzi et al., 2003; van Raamsdonk et al., 2005).

In the European STRATFEED project (G6RD-2000-CT-00414), an expert system named Animal Remains Identification and Evaluation System (ARIES) was developed to assist the microscopic detection of PAPs. Three modules for step-wise identification are being developed in it, together with a glossary, a gallery with additional series of images, a range of literature, and information on legislation. It also provides a full range of descriptions including shell fish and a range of plant parts and minerals that could be confused with animal material (Vermeulen et al., 2003, 2005).

Quantitative Analysis of PAPs by Microscopy

(Semi-) quantitative analysis of PAPs by microscopy is based on the presence of bone fragments. According to the guidelines mentioned in the EC/152/2009, the content of constituents of terrestrial animal products (percentage) can be estimated according to the following formula:

$$(S \times c)/(W \times f) \times 100$$

and the constituents of fish products (percentage) can be estimated as:

$$(S \times d)/(W \times f) \times 100$$

where S is the sediment weight (mg), c is the correction factor (%) for the estimated portion of terrestrial animal bone constituents in the sediment, d is the correction factor (%) for the estimated portion of fish bones and scale fragments in the sediment, f is the correction factor (%) for the proportion of bone in the original animal meal, and W is the weight of the sample material for the sedimentation (mg).

The correction factor c depends on the fraction of bones or other particles found in the sediment. There is however no explanation on how to evaluate this factor. In the guidelines, the assumption is made that "If the type of animal meal present in the sample is known, it

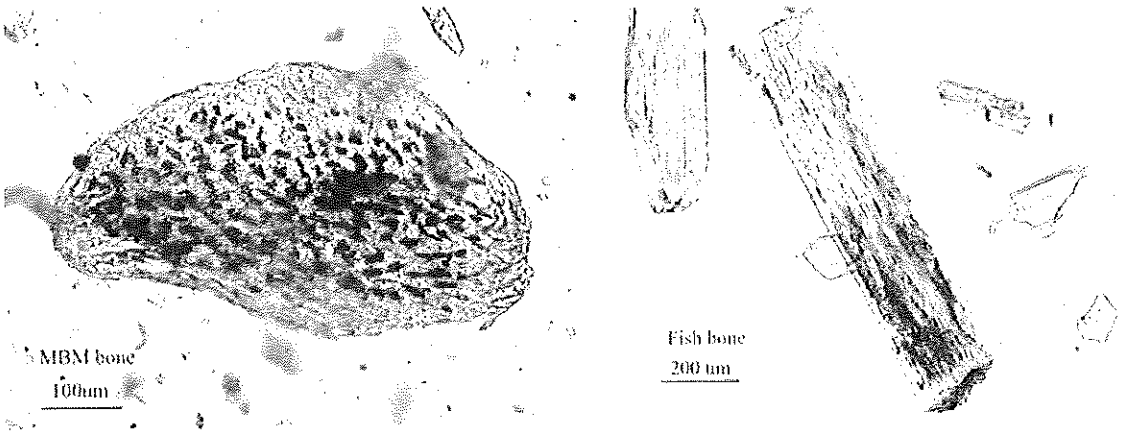

Fig. 2. Microscopic Pictures of PAPs (unstaining).

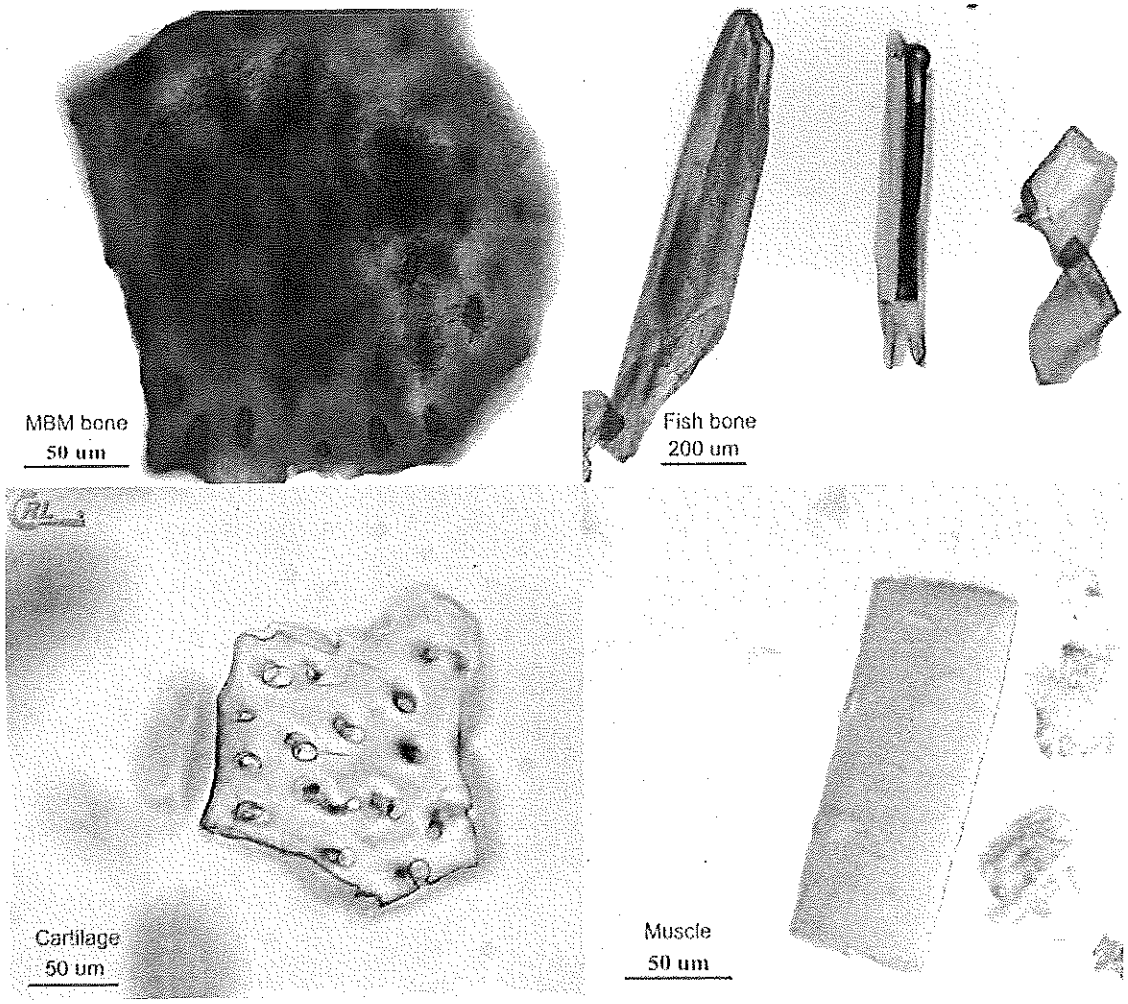

Fig. 3. Microscopic Pictures of PAPs (staining).

is possible to estimate the content." Theoretical indications of the percentage of bone in bone meal is between 50 and 60% (f factor = 0.5−0.6) and in the case of meat meals, between 20 and 30% (f factor = 0.2−0.3) (EC/152/2009). In practice, a much larger variation is found. As little as 10% of sediment can be found, even in the case of MBM. A large variation is also found in the percentage of bones in fish meal (Cruywagen, 1999).

The Community Reference Laboratory for Animal Proteins in feedingstuffs (CRL-AP) organized in 2006 an interlaboratory study for all National Reference Laboratories (NRLs) of the EU to investigate the robustness of the EC/126/2003 directive method regarding the quantitative evaluation of animal constituents in feeding stuffs. Results indicated that the variation between laboratories (reproducibility standard deviation) was not satisfying and recommended major improvements on more detailed instructions to be implemented in the present procedure (Veys and Baeten, 2007a). In 2007, another study was carried out to evaluate and validate a revised protocol for the quantitative analysis. This enhanced protocol included a determination of the d factor based on a grid counting process and the use of a standard calculation tool for the final estimation of adulteration by animal proteins. The grid counting principle is the correct application of the stereology method (Russ, 2005) for taking into consideration the volumes of the particles instead of their sole number. Results were appropriate for better standardization of measurements. The need of microscopists' experience and continuous training was highlighted in this study (Veys and Baeten, 2008).

THE ALTERNATIVE METHODS FOR DETECTING PAPs IN FEEDSTUFF

Polymerase Chain Reaction

The polymerase chain reaction (PCR) method is a revolutionary method developed by Kary Mullis in the1980s. It is based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. Tartaglia et al. (1998) firstly applied DNA detection method as an analytical tool to test for the presence of bovine-derived materials. By now, lots of literatures have been published about different targets and methods for PCR or real-time PCR on detection of PAPs in feed (Aarts et al., 2006; Frezza et al., 2003, 2008; Fumière et al., 2006, 2010; Prado et al., 2004, 2007). It is suggested that PCR method allows a rapid and sensitive detection of taxon-specific DNA-sequences from MBM. It could validate at 0.1% MBM in feed, below results are not always reliable (Cawthraw et al., 2009; Fumière et al., 2009; Yancy et al., 2009). PCR technique is powerful and flexible which can be applied to liquids, on samples of tiny particles and on animal by-products devoid of bones. It is also a relatively costly method and limited by heat-treatment of samples. Therefore, PCR is not suitable as a screening method, it could be preferred as a tool in combination with the other technologies to improve the detection of animal material and even identify it at species level or higher taxon levels (Gizzi et al., 2003).

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) technique is based on the analysis of specific proteins in feedstuffs. The most common approach is to develop immunoassays and making use of the specific antibody-antigen interaction (Hofmann, 1997). Several ELISA kits have been developed specifically devoted to the detection of meat and bone meals recently: the "Reveal for ruminant" test provided by Neogen Corp. (Lansing, MI), "Feedcheck" developed by Strategic Diagnostics (SDI-Newark, DE) for detecting PAPs from all animals and mammalian PAPs, and the inhibition ELISA for detecting ruminant PAPs proposed by AntibodyShop (Gentofte, Denmark) etc. These tests were subjected to many studies (Boix et al., 2004; Fumière et al., 2009; Klein et al., 2005; Myers et al., 2005; von Holst et al., 2006). It is generally rapid and easy to perform ELISA method as it does not need highly trained staff. However, the target analyte in ELISA analysis applied to the detection of PAPs is often not sufficiently characterized. A higher sensitivity and a better specificity need to be fulfilled. Therefore, ELISA method is suitable for the screening of a high number of samples for the presence of PAPs, combining its specific advantages with those of the other methods.

Near Infrared Spectroscopy

Near infrared spectroscopy (NIRS) as a rapid and cost-effectiveness method was first proposed in 1998 for the detection and quantification of MBM in compound feeds (Garrido-Varo and Fernandez, 1998). It is based on the absorption of light at selective wavelengths of electromagnetic spectrum by the molecules constituting the analyzed samples. It is necessary to analyze between 10 and 100 g of samples, which is enough to take into account the natural heterogeneity of samples and reduce the number of false positives. The limitation of NIR technique is a large range of reference samples analyses and high level of the limit of detection (exceeds 10 g/kg) (Baeten and Dardenne, 2002; Gizzi et al., 2003), so it cannot be used alone as legal evidence. Nevertheless it is practical for NIRS to play as the first line MBM screening technique in combination with other confirmation methods.

Near Infrared Microscopy

The potential of near infrared microscopy (NIRM) for feed authentication was studied firstly by Piraux and Dardenne (1999). NIRM method combines the analytical advantages of microscopy and spectroscopy technique. With a NIR microscope instrument, the infrared beam is focused through a dedicated microscope on each particle of a samples spread on a sample holder and the NIR spectrum is collected. It is demonstrated that NIRM method could detect MBM at a concentration as low as 0.1% in the raw and sediment fractions. However, reliable results require analyses of several hundreds particles and determining whether these particles are MBM particles or not by comparing their spectra with reference libraries. Thus the main limitation is the time consumption for the spectra collection in particle-by particle manner (Baeten et al., 2001, 2005a,b).

Advantages and Limitations of Light Microscopy on Comparison

As the only official method for the detection of PAPs in feedstuff in the EU, light microscopy has many advantages compared with the other four methods such as: very low level of false positive, accepted low detection limits, being unaffected by heat treatments of samples, simplicity if the operator has experience for identifying animal structures (Momcilovic and Rasooly, 2000). Meanwhile, light microscopy method has been indicated as having weakness of time consuming, needing skilled staff, lacking of animal or species specificity

and being unable to provide accurate quantification of animal material. It is revealed that it is difficult to distinguish bones of mammalian origin from those of poultry origin in the current practice (Gizzi et al., 2004). Its applicability for liquid samples and the use of toxic solvents have been cited as disadvantage as well (Gizzi et al., 2003).

CONCLUSIONS AND PERSPECTIVES

Light microscopy is a reliable and accepted method for detecting PAPs in animal feed, with the current level of positive detection being at or below a concentration of 0.1% of MBM. Yet the future intraspecies recycling ban requires not only a reliable distinction between the origins of animal meal at the level of vertebrate classes (mammal, avian and fish), but also at lower taxonomy levels, e.g., bovine versus porcine. The deeper taxonomic distinction is hard for microscopy technique, therefore the use of alternative methods should be considered. Also further knowledge should be acquired for discriminating between classes of terrestrial animals (mammalian versus avian). New characteristics and additional descriptions might be developed for the other particles such as striated muscles besides bone fragments. It was indicated that a so-called muscle ratio between number of striae per unit of the fiber width might give information to identify different species samples, and more details should be investigated (van Raamsdonk et al., 2004, 2005). Further research should be conducted on both the discriminative power and on the practical application.

Comparing the performance of different methods for detection of PAPs in feedstuff indicates that there is no ultimate approach to fulfill all the requirements. The advantages and disadvantages of the different approaches show that these methods are complementary rather than competitive. Therefore, a combination of different technologies could be a good approach for future support of the species-to-species ban and should be developed. For examples, a strategy combining the NIRM with PCR method developed by CRA-W. NIRM method detects and isolates the particles of MBM origin, and PCR technique could be used for the species identification; ELISA or PCR method combined with microscopic analysis can also be presented as good approach for support of the species-to-species ban. Light microscopy can be used as a screening method for the almost total absence of false negatives, with either ELISA or PCR as identification methods. The problem of false negatives when applying ELISA and PCR could then be avoided, and both of them can indicate the source of the animal proteins.

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