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Review

The potential of near infrared microscopy to detect, identify and quantify processed animal by-products

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Epidemiological studies have indicated that the most likely pathway of the infection of cattle with bovine spongiform encephalopathy (BSE) is feed-borne contamination with animal proteins. The enforcement of the ban on meat and bone meal in feed for farmed animals, including the "species-to-species" ban, is considered an important measure to prevent the spread of BSE. This review summarises more than ten years of work on species identification, quantification, comparison with optical microscopy, combination with polymerase chain reaction and detection based on chemometric decision rules and discriminant models. In the first part of the review, a summary is given of existing methods for the detection, identification and quantification of processed by-products of animal origin. In the second part, the possibilities offered by the near infrared microscopy technique for detecting, identifying and quantifying processed by-products of animal origin is reviewed. Work needed on enforcing the feed ban in terms of both routine and official control measures is also discussed.

Keywords: feed safety, near infrared microscopy NIR microscopy, bovine spongiform encephalopathy, BSE, processed animal proteins, PAPs, detection, identification, quantification

Introduction

Feedstuffs are composed of feed materials derived from products of vegetal, animal or mineral origin. They are used in their natural state or have been processed. In the case of feed materials of animal origin, the process of transforming animal by-products into valuable products [for example, meat and bone meal (MBM) and fat] is called "rendering". In EC Regulation 1774/2002,[1] these products are called processed animal proteins (PAPs), but only PAP material from category 3, which consists of animal by-products that are fit for human consumption, can be used to feed farm animals. Under this definition, PAPs do not include blood products, milk or milk-based products, colostrum, gelatine, hydrolysed proteins and dicalcium phosphate. Of all the materials listed in category 1 or category 2, MBM is one of the most important PAPs among the processed animal by-products derived from the processing because of its quantity and market value. MBM is defined in Commission Directive 98/67/EC as "the product obtained by heating, drying and grinding whole or parts of warm-blooded land animals from which the fat might have been partially extracted or physically removed". The product should be substantially free of hooves, horn, bristle, hair and feathers, as well as digestive tract content.[2] For more than 10 years, MBM has been the focal point of the control laboratories and management bodies responsible for addressing the bovine

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spongiform encephalopathy (BSE) epidemic. Epidemiological studies have shown that the most likely pathway of the infection of cattle with BSE is feed-borne contamination with animal proteins.[3,4]

The earliest case of BSE occurred in 1984 in the UK, with further BSE outbreaks occurring in the late 1990s.[5] The first BSE case in Asia was reported by the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) in 2001. The Organisation Internationale des Epizooties (OIE) reported that there were about 25 countries where cattle were infected with BSE. To date, there have been more than 190,000 reported cases of BSE in Asia, Europe and North America (http://www.oie.int).

For this reason, strict legislation has been enacted in Europe and elsewhere in the world to prevent MBM from entering ruminant feed.[3] The use of mammalian meat and bone meal (MMBM) to feed ruminants was banned throughout Europe in 1994; this was the first time that MMBM had been banned as a feed ingredient.[6] The discovery of BSE cases in animals born after the first ban, however, forced European authorities to extend and reinforce the partial feed ban. In 2000, the ban on PAPs in relation to farmed animals was extended for a defined period. Later, a permanent ban was introduced relating to the use of protein derived from mammals to feed ruminants and the prohibition was extended for farmed animals. Derogation was applied to the use of milk, milk-based products, colostrum, eggs, egg products, gelatine derived from non-ruminants and hydrolysed proteins derived from parts of non-ruminants and from ruminant hides and skins.[7] Derogation was also applied to the feeding of non-ruminant farmed animals with fish meal, dicalcium phosphate and tricalcium phosphate, as well as with blood products derived from non-ruminants.[8] Subsequently, the use of fish meal for young farmed ruminant species was authorised only for the production of milk substitutes.[9] The MAFF in Japan introduced a "complete feed ban" whereby all animal proteins, apart from milk protein, were prohibited in feed for ruminants, swine and poultry. Later, this ban was amended to allow protein derived from swine and poultry to be used in feed for monogastric animals on condition that these proteins were produced in mills where no ruminant materials were handled.[10] To date, there have been no BSE cases in China, but the country's Ministry of Agriculture has nevertheless enacted regulations to ensure the safety of the feed chain. These regulations state that: (1) PAPs, especially those derived from ruminants, are strictly prohibited in feed for ruminants;[11] (2) the importation of PAPs from countries with BSE cases is prohibited; and (3) the regulations are enforced to manage feed products of animal origin, to prevent contamination during production processes and to ensure the source and animal species of raw materials.[12] According to these regulations, MBM can still be used to feed monogastric animals in China.

As discussed earlier, the European policy was driven mainly by the goal to eradicate BSE in the European area. This explains why, after the partial ban in 1994, the European Commission then reinforced the ban by introducing an extended and permanent ban in 2001. The problem at that time was not so much the voluntary adulteration of the feedstuffs but rather accidental cross-contamination. About 90% of mills in Europe share facilities for producing both ruminant and non-ruminant feedstuffs, so cross-contamination at feed mills was highly probable.[13] Unintentional cross-contamination of ruminant diets with feed intended only for monogastric and poultry species was therefore thought to be the primary cause of the persistence of the BSE epidemic after the 1994 partial feed ban had been implemented.[13] Later, the Animal By-Products Regulation included text to regulate the use of animal by-products to feed the same species, thus preventing cannibalism.[1] To implement this, methods were needed for discriminating, at species level, the animal by-products found in compound feed. In order to avoid cross-contamination, it was essential to adopt appropriate strategies (including suitable analytical methods) during the production, transportation and storage of all raw materials and feedstuffs.

The European Food Safety Authority recently published a Scientific Opinion about the quantitative risk assessment of BSE in relation to a small amount of MBM. It concluded that the current global limit of PAPs detection in feed is still considered to be 0.1%, but it recommended continuing the development of analytical methods to improve this limit of detection (LOD) with regard to animal proteins in feed.[14]

Various analytical methods have been proposed to ensure effective control of the feed bans. In the next section of this paper, a summary is given of the existing methods for the detection, identification and quantification of processed by-products of animal origin. These methods include optical microscopy (OM), polymerase chain reaction (PCR), immunological techniques, near infrared (NIR) reflectance spectroscopy, near infrared (NIR) microscopy and NIR imaging. The paper focuses particularly on the possibilities offered by the NIR microscopy technique for detecting, identifying and quantifying processed by-products of animal origin. It summarises more than 10 years of work and discusses further studies required to implement the NIR microscopy method as part of routine and official control measures.

Overview of existing methods for the detection, identification and quantification of animal proteins in feed

Since the BSE crisis and the suspected link between PAPs and BSE, efforts have been made to develop existing and new methods of detecting the presence of animal products in compound feeds. This section summarises the main features of these methods and their main pros and cons. There are more details in recent reviews.[13,15,16]

Optical microscopy

Currently, MBM is detected mainly by OM. This involves the visual identification of specific morphological (for example, feathers in the detection of avian products) and histological features (for example, lacunae and canaliculae in the identification of bones). In order to perform an OM analysis, the

sample is ground (usually at 1 mm or 2 mm) and the particles are spread on microscope slides and observed. There are several sample preparation protocols, including the treatment of sample fractions with specific reagents for detecting specific particles of animal origin. These protocols allow, for example, the detection of muscle fibres, bones, feather or blood. According to the protocol, the analysis is performed on the sample either as it is or on the sediment obtained after a specific solvent has been used to extract the dense fraction of the sample (for example, in observation of bone and scales).[15,17] Figure 1 shows some specific morphological and histological features of animal proteins.

In Europe, the OM method is the only official method for determining constituents of animal origin in feedstuffs. It has been validated through several European inter-laboratory studies.[17,18] The method (including all the protocols for analysing specific particles) is described in Annex VI of the Commission Regulation 152/2009 laying down the sampling and analysis methods for the official control of feed.[19] This regulation has been in force since 26 August 2009 and replaced EC Directive 126/2003.[20] In practice, the detection of illicit ingredients of animal origin is driven mainly by the detection of bones and scales, which is performed on the sediment fraction obtained after decantation of the ground samples in tetrachloroethylene. In doing so, the dense fraction (i.e. particles with a density higher than 1.62) is obtained and the particles from this fraction are observed. For detecting bones, a trained analyst looks for specific bone features of lacunae and canaliculae. A lacuna is a cavity with a diameter of 10–50 µm where the osteocyte was located before being removed by the rendering process.

As described in the Commission Regulation 152/2009, the sensitivity of the method depends on the nature of the constituents of animal origin. The LOD has not yet been assessed; various studies have shown, however, that the LOD is less than 0.1% when bones are present in PAPs included in the feed.[21,22] The LOD of the method when there are only muscle particles is higher than 0.1% (it is thought that the LOD for this specific detection is about 1%).[16,17] The LOD of the OM method is mainly affected by the nature of the constituents of animal origin and by the skills of the operator.

The ability of OM to identify the species origin of particles is limited. As explained earlier, fish material can be discriminated by detecting the presence of scales or bones with specific features at the level of the particles and lacunae (the shape and distribution of the lacunae varies, depending on the animal source). This discrimination is not possible, however, in the observation of muscle fibres, for example. Nevertheless, it has been reported that particles from specific parts of bones from terrestrial animal are seldom confused with fish particles.[23] The discrimination among other animals is limited to the detection of avian material by the presence of feathers. The method described in 152/2009 (see Point 7 in Annex VI) also includes a protocol for quantification. Recent inter-laboratory studies,[24] however, have demonstrated an inability to reproduce this quantitative protocol, thus preventing its use in official controls.

Polymerase chain reaction

Much work has been done on developing polymerase chain reaction (PCR)-based methods for the detection and identification of DNA in animal origin. These methods are based on genetic amplification, which is considered as one of the most efficient ways of detecting specific DNA targets. The most popular protocols for amplifying DNA are those using PCR. PCR methods have a high forensic value because they are based on researching specific DNA sequences characteristic of the taxonomy level (for example, mammal, ruminant) or the species level (for example, bovine, pig). Among the PCR methods proposed, most did not transfer successfully to another laboratory or pass evaluation by an independent laboratory. It is essential to use multi-copy targets (for example, from mitochondrial DNA) and targets of reduced length (ideally, about or fewer than 100 bp because animal proteins are subjected to an aggressive rendering process which causes degradation of DNA.[25,26] Several PCR methods have passed in-house validation successfully, however, and were positively evaluated in a study conducted by the European Joint Research Centre.[27,28] These methods allow species-specific detection of fish, cattle, pigs, poultry and sheep. This detection can be done either on the sample as it is or on the sediment. Some methods have been accredited in line with ISO 17025 and are used in addition to the official method (i.e. OM). Today, some non-European countries use PCR methods for official control measures. When the compound feed includes authorised animal by-products (for example, fat, milk or egg by-products), the detection by PCR of animal DNA is confused in assessing the presence of forbidden materials (for example, pig, bovine or avian).

Figure 1. Specific morphological and histological features of animal proteins.

As the DNA content in PAPs will depend on the source and composition of the material used to produce it and the rendering process applied, PCR methods cannot be used for quantification purposes.[16]

Immunological methods

There are several immunochemical techniques. In the detection of PAPs, however, the most common techniques used in developing detection methods are enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays (dip sticks).[16] Ansfield *et al*.[29] developed an immunoassay method based on the use of antibodies against a thermostable antigen. It is able to detect the presence of ruminant and porcine proteins in feedstuffs and has an LOD of about 0.1%. It has not yet been validated through an inter-laboratory study.[15,18,29,30] ELISA kits have been developed that show promising results for detecting the species origin of PAPs.[16,31] There are several lateral flow dipstick-based methods that have the potential to detect PAPs.[31,32] They enable PAPs from ruminants or mammals to be detected. The LOD of these methods is about 0.5–1% and limitation has been reported when some of these methods were used for detecting ruminant PAPs in pig material.[33,34] These methods are fast, easy to implement and suitable for identifying specific PAPs.

Near infrared reflectance spectroscopy

NIR spectroscopy is the most widely used non-destructive method in the feed industry to determine qualitative parameters of feed ingredients and feedstuffs. The high sample throughput of the method, its capacity to determine a large range of parameters[35] (from major constituents to criteria such as digestibility) in a single analysis and the possibility of building a network of spectrometers make this technique very attractive for the feed sector. The fact that it can also be used online in a feed production plant adds to its attraction. The principle of this method is based on the absorption of light with near infrared wavelengths by the molecules that make up the sample. Cozzolino and co-researchers explored the possibility of using NIR spectra for the characterisation of fish-meal samples.[36] The absorption bands at 1490 nm (6711 cm^{-1}) and 1944 nm (5144 cm^{-1}) were associated with water content, whereas the bands at 2060 nm (4854 cm^{-1}) and 2168–2180 nm (4613–4587 cm^{-1}) showed a high correlation with crude protein. Fat content was related to absorption regions around 1700–1730 nm (5882–5780 cm^{-1}) and 2300–2310 nm (4348–4329 cm^{-1}). Several papers have shown the potential of NIR spectroscopy to detect PAPs of terrestrial origin in feedstuffs[37–40]and in fish meal.[41–43] The NIR technique has the advantage of being able to analyse both ground and unground samples. It is a fast and cost-effective technique that requires a low level of expertise.[44] The largest study conducted on this topic at the European level was done within the framework of the STRATFEED project.[45,46] In this EC project (www.stratfeed.cra.wallonie.be), a network of five spectrometers was set up and global equations were developed. The results from this project indicated that NIR spectroscopy could provide the feed industry with a fast screening method for detecting the contamination of compound feed with processed animal by-products. STRATFEED also set the LOD in the network at 1–1.5%, which is not sufficiently accurate to be used as evidence in cases of fraud or accidental contamination. Nevertheless, this limit is interesting for the auto-control performed at feed mill level.

Recent studies have reported that NIR spectroscopy can be used to identify and quantify the animal species (poultry by-products, pig, cattle, ruminant and non-ruminant) in PAPs. The models developed enable the unequivocal classification of poultry by-product meal and pork meal.[47,48] Until now, no NIR method has been validated by an inter-laboratory study following international guidelines.

Near infrared microscopy: principles and instrumentation

With NIR spectroscopy, the detection of PAPs in feedstuffs is based on a single NIR spectrum corresponding to the mean of several scans obtained from a representative portion of the sample. It means that the spectral information from the specific absorption of the radiation by PAPs is diluted by the absorption of the radiation of all the feed ingredients. Techniques able to collect the spectrum of individual particles from samples would be required to detect specific particles of determined origin. In this instance, PAPs detection would not be performed through the analysis of a single spectrum, but through the analysis of hundreds or thousands of spectra from individual particles. This can be done using an NIR microscope. This hyphenated instrument includes a classical NIR spectrometer coupled with an optical microscope in which the optics have been adapted to NIR radiation. NIR microscopes allow the spectra to be collected from extremely small sample areas (typically, 50 µm × 50 µm or less, depending on the instrument and the configuration). Usually, the instrument includes a charge coupled device camera and a viewing system that magnifies the visible light image of the sample, allowing the user to visualise it and to position an infrared beam on the sample area of interest using a motorised stage. Using the microscope pointer, the infrared beam is focused on each point of interest and the near infrared spectrum is collected.[49]

The principle of identifying PAPs by NIR microscopy is similar to the European official method. Instead of visibly observing the morphological and histological features of particles, however, infrared absorbances of the compounds making up the particle are used. OM is based on optics, visual detectors (i.e. the eyes of the analyst) and a sophisticated trained expert system (i.e. the brain of the analyst). NIR microscopy is based on optics, NIR detectors and mathematical models (i.e. calibration equations). In fact, NIR microscopy combines the analytical advantages of OM and NIRS. NIR microscopy requires a low level of expertise, it is a non-destructive method and has a detection limit lower than 0.1%. Moreover, it has a low level of false negatives and a high repeatability and it is independent of the feed matrix used.[50] NIR microscopy-based methods make the assumption that each particle is made up of a single feed ingredient. Figure 2(a) shows a commercially

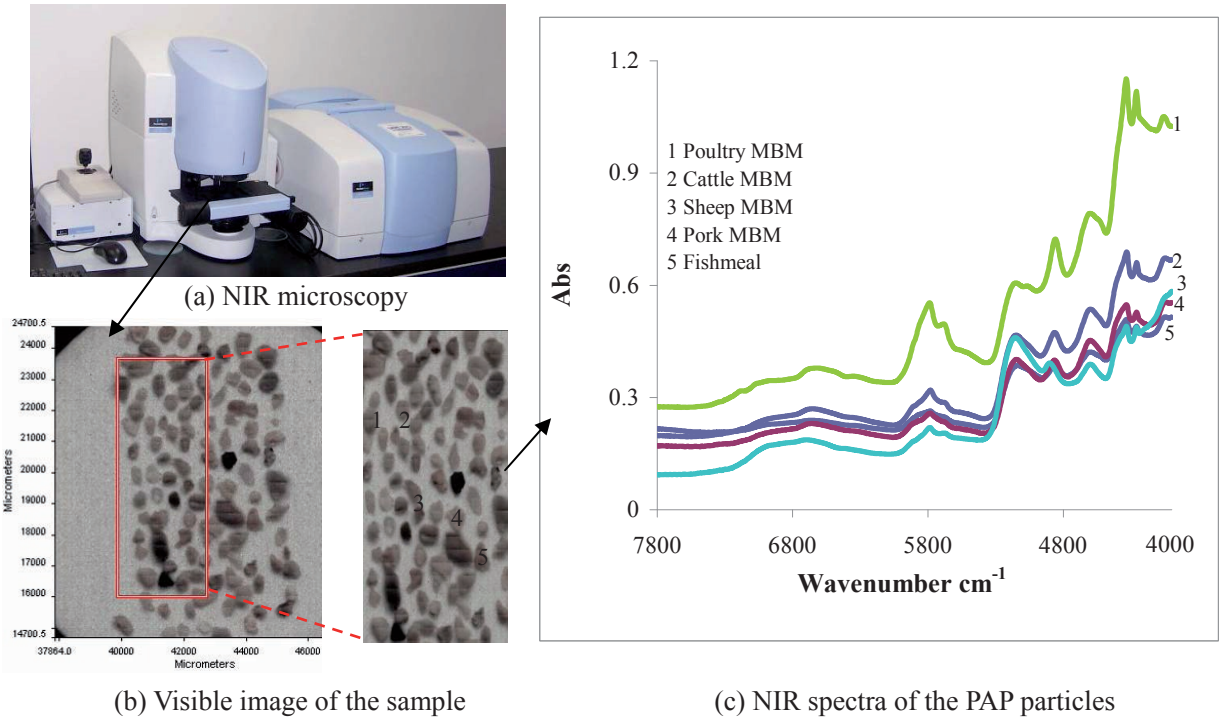

Figure 2. (a) Perkin–Elmer FT-NIR microscope; (b) visible image; and (c) NIR spectra of the PAPs particles.

available instrument from the Perkin–Elmer Company. Figure 2(b) shows the visible image of a sample displayed on a reference surface (spectralon). The spectra in Figure 2(c) were collected from different particles in reflectance mode, derived from five animal origins (fish meal, poultry MBM, pork MBM, cattle MBM and sheep MBM). The spectrum characteristics obtained by NIR microscopy correspond to those of NIR spectroscopy. The rich absorption around 5800–4000 cm^{-1} is related to crude protein and fat. Examples of various kinds of animal by-product particles (i.e. muscle, feathers, hair, horn, teeth, bones, scales, blood products, milk, milk-based products, colostrum, gelatin, hydrolysed proteins, dicalcium phosphate, fat and egg by-products) have been described in the literature.[51]

Evolution of the near infrared microscopy methodology

Detection of processed animal proteins

Several feasibility studies based on the application of NIR microscopy to detect PAPs have been reported over the last ten years. These applications are summarised in Table 1.

Detection by discriminant equations

In 1999, Piraux and Dardenne published the first research paper demonstrating the potential of NIR microscopy for detecting unauthorised animal ingredients in compound feed.[52] Their work involved collecting 1740 particles from 56 raw materials (allowed at that time) for feeding ruminants, including fish meal, peas, manioc, wheat, blood meal, rape-extracted oil cake, corn, maize gluten feed, maize germ oil cake, soybean, flax, alfalfa and milk by-products. A total of 1291 particles from 43 animal meal products (forbidden at that time), including MBM, meat meal, ground bones, feather meal and poultry by-products were obtained. An NIR Perkin–Elmer microscope was used to scan the particles in reflectance mode with an aperture size of 50 µm × 50 µm. The spectra were collected from 1112 nm to 2500 nm and resulted from an average of 100 scans. These spectra were pre-processed using standard normal variate and detrend (SNVD), as well as first derivative as the mathematical treatment. An artificial neural network model was used to discriminate the "allowed" particles from the "forbidden" ones. The model was applied to an independent validation set containing 1872 particles. The results of the discriminant analysis indicated that it was possible to detect MBM particles in feedstuffs with a success rate greater than 99% by NIR microscopy (i.e. a total of false negative and false positive results of less than 1%). The results showed that it was possible to detect forbidden animal particles in a ground compound feedstuff with an overall error rate of 0.64% (the average value of the rate of allowed particles misclassified as forbidden particles and the rate of forbidden particles misclassified as allowed particles). The study also showed that if the MBM proportion in a compound feedstuff was low, it was necessary to scan a large set of particles to find at least one MBM particle. For example, for a feedstuff

Table 1. Detection of processed animal proteins by near infrared microscopy.

Study purposes	Training set	Validation set	Model; method	Success rate	Raw/sediment fraction	Importance and/or weakness	Ref.
To detect unauthorised animal ingredients in compound feed	1740 particles (780 for calibration and 960 for validation) from 56 allowed raw materials (fish meal, peas, manioc, wheat, blood meal, rape-extracted oil cake, corn, maize gluten feed, maize germ oil cake, soybean, flax, alfalfa and milk by-products); 1291 particles (379 for calibration and 912 for validation) from 43 forbidden animal meal products (MBM, meat meal, ground bones, feather meal and poultry by-products)		Discriminant equation; ANN	>99% for both allowed and forbidden particles (validation)	Raw	This study for the first time reported the NIR microscopy method for feed inspection; There are no adulterated samples to validate the discriminant equation; Much more restrictive standard of detecting rules will be required to strengthen confidence in the classification	52
To detect animal ingredients in compound feed	7492 vegetal particles; 2484 animal particles	48 validation samples were prepared using 24 compound feeds spiked at various percentages (0.5–8%, w/w in 0.5% intervals) with eight MBM products	Discriminant equation; PLSDA	95% and 94.3% for the vegetal and animal particles (calibration) one sample was detected as negative (validation)	Raw	The detection result depends on the degree of homogeneity of the sample and the number of particles analysed; In order to strengthen confidence in the classification, the results of different equations to decide in which group a particle spectrum should be taken into account	51
To detect MBM in animal feeds	2229 animal particles (i.e. pigs, poultry, sheep, cows and a mixture of these); 1556 plant-based feeds particles (barley, maize, soybean, wheat, etc.)	(1) 18 compound feeds, including 10 MBM-free feedstuffs for livestock and eight pet foods containing 26–35% MBM (2) five compound feeds containing MBM (1%, 0.5%, 0.02%, 0.25%) and four containing different blood meal percentages (from 0.02% to 0.3%)	Discriminant equation; PLSDA	(1) Four false positive samples (maybe include animal fat or blood) (2) Validation result need to be confirmed by repeatability	Raw	This paper did not report repeatability data; Consideration about the possibility to detect at a level of 0.02% by analysing only 500 particles	55

Table 1 (continued).Detection of processed animal proteins by near infrared microscopy.

Study purposes	Training set	Validation set	Mode method	Success rate	Raw/sediment fraction	Importance and/or weakness	Ref.
To establish the suitability of NIR microscopy for detection of MBM by determining the sensitivity and specificity	4716 particles of animal origin; 3286 particles from compound feed	15 samples, both non-adulterated and adulterated with MBM at 0.05%, 0.1%, 0.5% and 1%, were used to study the repeatability. 48 spiked samples, obtained by mixing 24 feedstuffs with eight MBM products at different levels (0.5–8%, w/w in 0.5% intervals), and 24 compound feeds samples were used to study the reliability of the method	(1) Discriminant equation; PLSDA (2) Decision rule; Visual bservation	Success rate identifying animal particles was 97.5% by PLSDA equation, and 96.6% by decision rule (calibration) no false positive or false negative results (validation)	Sediment	The repeatability and reliability of this discriminant model were validated; Further research for decision rules will be needed to distinguish more easily and improve the accuracy	54
To establish the LOD and repeatability of NIR microscopy discriminant model	NA	A sample spiked at 0.1% with MBM was analysed 10 times	Discriminant equation; PLSDA	No false negative results (validation)	Sediment	The LOD and repeatability of the method were validated	50, 51
To develop appropriate decision rules and demonstrate the transferability	NA	20 samples with different MBM percentages (0–8%) were analysed	Decision rule; visual observation	No false positive or false negative results (validation)	Sediment	Appropriate decision rules were developed and validated; The transferability of decision rules was demonstrated by three labs; Further work should be launched to species identification and on quantification of PAPs	56

*Not available.

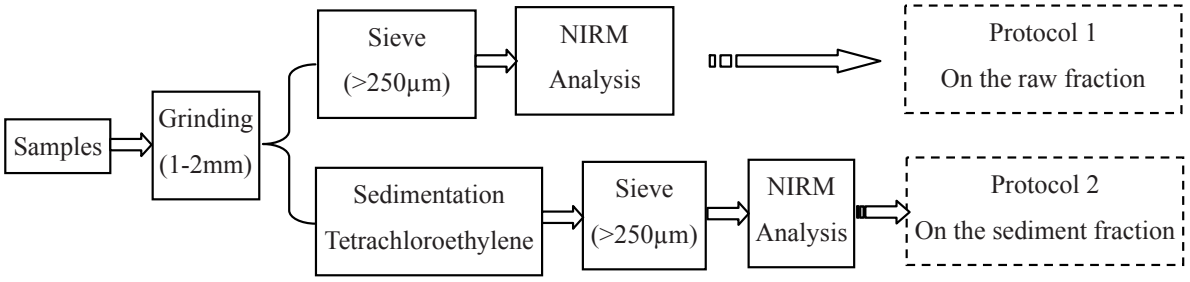

Figure 3. Schematic presentation of the two NIR microscopy protocols developed and validated in the STRATFEED project.

sample containing 0.5% MBM, in order to observe at least one MBM particle with a probability of 95%, about 1000 particles need to be analysed.[52,53] This study demonstrated for the first time that the NIR microscopy technique was suitable to address issues related to feed inspection. However, additional works on adulterated samples to validate the discriminant equations, as well as on the rules to decide in which group a particle spectrum should be placed, had to be performed.

Based on this preliminary research, the method has been developed and validated in-house within the framework of the European STRATFEED project. In this project, two protocols were developed (Figure 3). For the analysis of the raw fraction (non-sediment), the detection was based on the presence of various particles (for example, muscle and feathers, as well as those found in the sediment fraction) of animal origin present in the sample. For the analysis of the sediment fraction, however, detection was based only on the presence of specific animal particles (for example, bone, scales, cartilage).

The spectra of particles were collected from 1112 nm to 2500 nm in reflectance mode with an aperture size of 50 µm× 50 µm. Then a large spectral library was constructed, including more than 20,000 spectra from feed ingredients and compound feed. Based on the results of a principal component analysis, vegetal and animal particle subspaces were defined and then the animal particle sub-spaces were divided into fish particles and terrestrial particles. Finally, the terrestrial particle sub-space was sub-divided into poultry particles and mammal particles. Various supervised methods, including partial least squares discriminant analysis (PLSDA), artificial neural networks and soft independent modelling of class analogy (SIMCA), were also tested in order to build the discriminant equations.[50,51,53,54] The SIMCA method constructs a principal component analysis model for each individual group of interest and the allocation of new samples is based on the computing of distances to each model. The PLSDA method constructs a regression model between the spectral information and a matrix of dummy variables representing the different groups of interest. The artificial neural network technique processes information in a similar way as the human brain does. A network is composed of a large number of highly interconnected processing elements (neurons) working in parallel to solve a specific problem. It is a learning algorithm, i.e. before using a network for prediction it must be trained with known data. This is necessary to ensure that the artificial network provides useful results. While learning, the artificial network compares its output with observed (known) output values of learning data.

Near infrared microscopy analysis on the raw fraction

The detection of animal particles in the sample (as it was) was achieved by analysing 7492 vegetal particles and 2484 animal particles in reflectance mode with an aperture size of 50 µm × 50 µm. The samples were selected to cover the full diversity of feed ingredients used in formulating feedstuffs. In order to construct the discriminant equation, the PLSDA algorithm was used. Up to 95% of the vegetal feed ingredient particles and 94.3% of the animal particles were correctly classified. In order to test the mathematical models constructed, 48 validation samples were prepared using 24 compound feeds (destined for feeding cattle, goats, poultry, pigs and rabbits) spiked at various percentages (0.5–8%, w/w in 0.5% intervals) with eight MBM products. A randomised factorial design was used. Between 141 and 710 particles from the validation samples were analysed. Of the 48 validation samples, 47 were shown to be positive for the presence of MBM. Only one sample containing 0.5% MBM was erroneously detected as negative.[51] The detection ability depends on the degree of homogeneity of the samples and on the number of particles analysed. In this study, a new strategy was proposed to strengthen confidence in the classification results. Considering the results from different equations, it is decided in which group a particle spectrum should be placed. If a particle belongs to the animal group, it should be correctly discriminated by both the vegetal vs animal equation and the vegetal vs terrestrial animal (or fish) equation simultaneously.

De la Roza-Delgado *et al*. have also worked on the detection of animal particles in animal feeds.[55] They used 2229 particle spectra of animal origin (i.e. pigs, poultry, sheep, cows and a mixture of these) and 1556 spectra from plant-based feeds (barley, maize, soybean, wheat etc.) in order to establish discriminant equations by using PLSDA as the chemometric tool. The best results were obtained by applying SNVD and first derivative as pre-treatments. They used 18 compound feeds, including 10 MBM-free feedstuffs for livestock and eight pet foods containing 26–35% MBM, to validate the discriminant equation. No false positive or false negative results were

detected. They reported that the protocol developed could be used to detect the presence of MBM at concentrations as low as 0.02%, with 100% correct classification determined by the analysis of a validation set, including five compound feeds containing ≤1% MBM (1%, 0.5%, 0.02%, 0.25%) and four containing different blood meal percentages (from 0.02% to 0.3%). However, their paper did not report a repeatability study of the proposed protocol that would allow them to draw this conclusion. The protocol is based on the analysis of 500 individual particles, meaning that if the sample is perfectly homogeneous (in terms of particle distribution, density and size) the probability of detecting at least one animal particle in a sample adulterated at 0.02% is 0.09%. More than 15,000 particles would have to be analysed if a 95% level of confidence were to be reached with samples spiked at 0.02%.

Near infrared microscopy analysis on the sediment fraction

The protocol based on the analysis of samples as they are (non-sedimented samples) has demonstrated the potential of NIR microscopy to detect MBM at levels as low as 0.5%. In order to reduce the measuring time and the LOD, the addition of a sedimentation step before NIR microscopy analysis was proposed.[50,51,54] The protocol developed is fairly simple and very similar to the one used in the European official OM method (Annex VI in EC Regulation 152/2009). Sediment analysed by the official method is based on the principle of concentration of the bone fraction using a high-density solvent (i.e. tetrachloroethylene). In order to perform the discrimination, Baeten *et al*. constructed a PLSDA discriminant model with 8002 spectra scanned from the sediment of 27 meals of animal origin (including cattle, pig, sheep and poultry) and seven MBM-free compound feeds.[54] Some 97.5% of the animal particles were classified as animal, with a 95% confidence interval (twice the standard deviation). The misclassification ratio for animal particles was 0.064%. Two non-animal particles were wrongly classified in the animal class (meaning a ratio of 0.061% misclassification). Some 15 samples, both non-adulterated and adulterated with MBM at 0.05%, 0.1%, 0.5% and 1%, were used to study the repeatability. All samples containing MBM were correctly classified as "positive" and all samples free of MBM were correctly classified as "negative". Some 48 spiked samples, obtained by mixing 24 feedstuffs with eight MBM products at different levels (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, 6%, 6.5%, 7%, 7.5% and 8%) and 24 compound feeds samples were used to study the reliability of the method, including a sedimentation step on a wide range of MBM-free feeds. As a result, the 48 spiked samples were correctly detected as positive and the 24 blank feeds were correctly detected as negative. The LOD and repeatability of this discriminant model were validated. A sample spiked at 0.1% with MBM was analysed 10 times. All the replicates were detected as positive in terms of the presence of bones in the sample.[51] The analysis of the sediment fraction by NIR microscopy is an attractive and powerful method for detecting low levels of MBM in feedstuffs. With a protocol focused on the sediment part of the sample, NIR microscopy can be used to detect MBM in feed at levels as low as 0.05%.[54]

In feed control, a crucial aspect is the transferability of the developed methods from the developing laboratory to another laboratory. Within the framework of the STRATFEED project, it was decided to analyse blind samples in two laboratories that had not participated in developing the protocol. The samples were randomly analysed and the results showed that the NIR microscopy method based on the sediment fraction could be easily transferred to another laboratory and still return a false negative value of less than 5%.[51]

Detection by decision rules based on visual observation

Decision rules were developed based on absorbance values [log(1/*R*)] at different wavelengths of the spectrum from sediment particles in order to identify their animal origin. Baeten *et al*. proposed a decision rule using absorbance values at three wavelengths (i.e. 1944 nm, 2060 nm and 2148 nm) from the first (*d1*) and second (*d2*) derivative spectra to decide whether particle *i* belonged to the animal group or not.[51] The wavelengths were chosen as representative of the MBM spectra. The absorption bands at 1944 nm, 2060 nm, and 2148 nm were associated with water, protein and starch, respectively. A particle *i* belonged to the animal group if $d1(i,2148) > 0.001$, $d1(i,1944) > -0.001$, $d1(i,1944) < 0.003$ and $d2(i,2060) < 0$.[54]

von Holst *et al*. proposed a modification of these decision rules for analysing particle spectra from the sediment.[56] The decision rules are based on three criteria: (1) the presence of maxima in the 1920–1960 nm, 2030–2070 nm and 2150–2200 nm regions; (2) the presence of minima in the 2010–2030 nm (*y1*), 2070–2150 nm (*y2*) and 2210–2250 nm (*y3*) regions; and (3) fulfilling the formula [(absorbance around *y1* + absorbance around *y3*)/2] > absorbance around *y2*. This last criterion depends to a great extent, however, on the slope of the spectrum and this can lead to mathematically classifying as animal a sample that visually is not. In order to solve this problem, the third criterion can be generalised (even for non-sediment samples) using the following equation: $\{\text{absorbance [line segment } (y1\text{–}y3)]_{\text{wavelength defined by } y2}\} > \text{absorbance } (y2)$ (Figure 4). Based on the decision rules, 20 samples with different MBM percentages (0–8%) were analysed and no false positive or false negative results were observed.

von Holst *et al*. reported also the results of a transferability study in which these decision criteria based on the visual inspection of the NIR spectrum were applied to measurements obtained by three independent laboratories.[56] The three laboratories [Walloon Agricultural Research Centre (CRA-W), Institute for Health and Consumer Protection (IHCP), and Institute for Reference Materials and Measurements (IRMM)] involved in the transferability study were equipped with a Fourier transform (FT) near infrared microscope from the same company (Perkin–Elmer), but the model and specification were different; at CRA-W a Spectrum IdentiCheck FT-NIR system and Auto Image microscopy, at IHCP a FT infrared

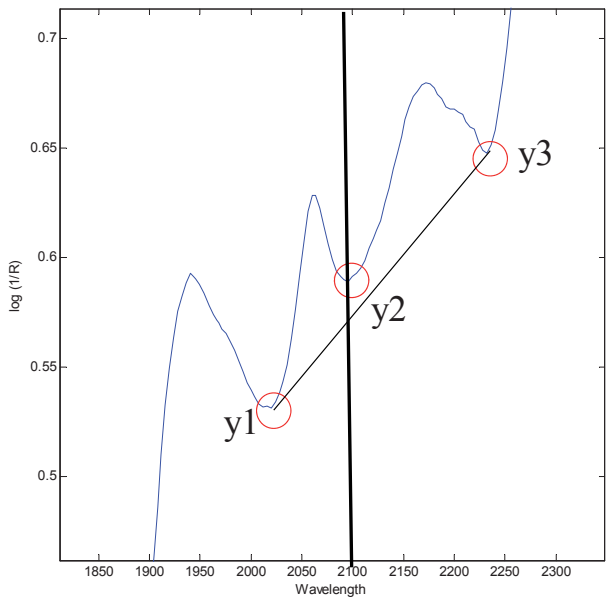

Figure 4. Spectral conditions to be fulfilled by a spectrum from a particle of animal origin (adapted from von Holst *et al.*[56]).

Spectrum 2000 system and Auto Image microscopy and at IRMM a Spectrum One NTS system and Spotlight microscopy. Each laboratory analysed the same 20 samples with and without different levels of MBM (0–8%). Staff at CRA-W and IHCP analysed 3500 particle spectra, respectively, and staff at IRMM scanned about 5500 spectra. All positive and negative samples were correctly identified at each of the three laboratories based on the proposed decision rules.[56]

In summary, NIR microscopy is an interesting alternative method with many advantages, such as being an objective, reliable, sensitive and highly selective technique. However, the detection result greatly depends on the homogeneity of the samples and the number of particles analysed. A large number of particles need to be analysed when the content of animal ingredients in an adulterated sample is too low.

Species identification of processed animal proteins

Several studies have been carried out to assess the potential of NIR microscopy for species identification (Table 2). This issue is essential in order to have suitable methods that allow the lifting of the total ban and permit intra-species recycling (for example, use of pig meal for feeding poultry). These studies include analyses on both raw and sediment fractions.

Near infrared microscopy analysis of the raw fraction

Baeten *et al.* were the first to study, on a large scale, the potential of NIR microscopy methodology for identifying the species origin of an animal particle.[51,53] In order to achieve this goal, 7492 vegetal particle spectra and 2484 animal particle spectra (including 1595 terrestrial animal particle spectra, 889 fish meal particle spectra, 505 poultry by-product particle spectra and 1090 bovine and pig meal particle spectra) were used to construct species identification equations using the PLSDA algorithm (Table 3). Correct classification rates of 95.0%, 94.3%, 95.5%, 90.0%, 84.1% and 90.1% were obtained, respectively, for the vegetal, animal, terrestrial animal, fish, poultry by-product and pig/bovine particles. If particle *i* belonged to the terrestrial animal group, it should be discriminated correctly by Equations (1), (2) and (4). If particle *i* belonged to the fish meal group, it should be discriminated correctly by Equations (1), (2), (4) and (5). If particle *i* belonged to the poultry by-product or the bovine and pig MBM groups, it should be discriminated correctly by Equations (1), (2), (3) and (4). This strategy strengthens confidence in the classification.

Ten fish meal samples spiked with MBM at various levels (3%, 6% and 9%) were used to detect terrestrial animal by-products in fish meal by analysing about 600 particles of each sample. All the spiked samples were shown to be positive in terms of the presence of terrestrial animal by-products.[51] However, the discriminating power of NIR microscopy for the detection of traces of PAPs at higher taxonomic levels should be proved by further research.

Pérez-Marín *et al.* developed a methodology using only a library of animal meal by-products for identifying terrestrial MBM and fish meal.[57] The training database comprised 11,727 spectra of particles coming from pure terrestrial meal (eight bovine, four ovine, 40 porcine, and 41 avian samples) and 5843 spectra of particles of 65 fishmeal samples. The method was based on a two-stage strategy. In the first stage, animal particles were identified by using either the *k*-nearest-neighbour (*k*NN) method or SIMCA. In the second stage, *k*NN was used to discriminate between terrestrial and fish particles. *k*NN with second derivative spectra and five neighbours correctly classified 98.5% when using cross-validation. Some 20 experimental mixtures of fish meal spiked with various bovine meals (2.0–16%) were used to validate the method. About 200 particles per sample were analysed by NIR microscopy. The presence of terrestrial animal by-products was correctly detected in 20 external validation samples. Furthermore, 20 commercial compound feeds were used to validate the constructed models. These samples included pure compound feeds, compound feeds adulterated with 2.5% or 3.0% fishmeal and compound feeds adulterated with various terrestrial MBM (14.3–29.4%). The models worked perfectly for the pure compound feeds; however in the case of adulterated samples, although all the terrestrial MBM was detected, more than half of the identified animal particles were wrongly classified as fish. This result indicated that the accuracy of identifying fish meal and terrestrial MBM needs to be improved.

Fumière *et al.* proposed combining NIR microscopy and real-time PCR to authenticate, at species level, the presence of animal particles (see the section "Combining near infrared microscopy and other analytical technologies").[58,59] To implement this, an identification of the animal particles was done initially using NIR microscopy. After this analysis, each suspect particle was individually and carefully transferred in a vial for

Table 2. Species identification of processed animal proteins by near infrared microscopy.

Study purpose	Training set	Validation set	Model; Method	Success rate	Raw/ sediment fraction	Importance and/or weaknesses	Ref.
To demonstrate the potential of NIR microscopy for species identification of PAPs	7492 vegetal particles and 2484 animal particles (including 1595 terrestrial animal particles, 889 fishmeal particles, 505 poultry by-product particles and 1090 bovine and pig particles)	10 fishmeal samples spiked with MBM at various levels (3%, 6% and 9%)	Discriminant equations; PLSDA	95.5%, 90.0%, 84.1% and 90.1% for the, terrestrial animal, fish, poultry by-product and pig/ bovine particles (calibration) no false negative results (validation)	Raw	Hierarchy equations were established, which strengthens confidence in the classification; The spiked samples needed to have a good level of homogeneity; The discrimination power of NIR microscopy for the detection of traces of PAPs at a higher taxonomic lever should be validated by further researches	51
To discriminate terrestrial MBM from fishmeal	11,727 spectra from terrestrial meals and 5843 from fishmeal	20 experimental mixtures of fishmeal spiked with various bovine meals at various levels (2.0–16%); 20 commercial compound feeds, including pure compound feeds, compound feeds adulterated with 2.5% or 3.0% fishmeal, and compound feeds adulterated with various terrestrial MBM at different levels (14.3–29.4%)	Two-step strategy: (1) animal particles are identified using a global (SIMCA) or a local (*k*NN) distance measure; (2) terrestrial and fish particles are discriminated by *k*-nearest-neighbours (*k*NN)	98.5% (calibration) No false negative results (validation, for mixtures of fishmeal spiked with bovine meals) More than half of the identified animal particles were wrongly classified as fish (validation for mixtures of compound feeds spiked with terrestrial	Raw	This study only use a library of animal meal by-products for identifying terrestrial MBM and fishmeal; This result indicates the accuracy of identifying fishmeal and terrestrial MBM need to be improved	57

Table 2. Species identification of processed animal proteins by near infrared microscopy.

Study purpose	Training set	Validation set	Model; Method	Success rate	Raw/ sediment fraction	Importance and/or weaknesses	Ref.
To discriminate cattle from pig particles	738 particles (278 cattle and 460 pig particles)	NA	Discriminant equations; PLSDA and SVM	97.3% for SVM and 99% for PLSDA (calibration)	Raw	With the help of PCR, the species-specific NIR microscopy spectral database can be established; The combination of NIR microscopy and PCR benefits from the advantages, and overcomes the limitations of both methods	58, 59
To identify mammalian MBM and fishmeal	1380 particles with 420 fishmeal particles	The validation set included 630 particles with 90 fishmeal particle	Discriminant equations; SVM	100% (calibration) 95% (leave-one-out cross validation) 95.5% (validation)	Sediment	Further developments will focus on extending libraries with more fish and MMBM particles; This result indicates the accuracy of identifying fishmeal and mammalian MBM need to be improved	60

NA, not available.

Table 3. Description of the equations constructed for species identification.

Equation		Groups					
N°	Discrimination	Vegetal	Animal	Terrestrial animal	Fish	Poultry	Bovine and pig
1	Vegetal/animal	•	•	•	•	•	•
2	Terrestrial animal/fish			•	•	•	•
3	(Bovine and pig)/poultry					•	•
4	Vegetal/terrestrial animal	•	•	•	•	•	•
5	(Vegetal and terrestrial)/fish		•		•		
Number equations used		2	3	3	4	4	4

Data are taken from Baeten *et al.*[50] and De La Rosa Delgado *et al.*[55]

PCR analysis, and its origin (animal or vegetal) was predicted using existing discrimination models. The NIR microscopy spectra of 738 particles (278 cattle and 460 pig particles) were obtained. After the NIR microscopy analysis, the MBM particles were recovered and analysed by real-time PCR to confirm their species origin. Only the spectra corresponding to particles giving a clear PCR result were implemented in the spectral databases used to develop discriminant models. Support vector machines (SVMs) and PLSDA models were constructed using the training dataset in order to discriminate between cattle and pig particles. When the models were applied to an independent dataset, both procedures correctly classified more than 97% of the samples. SVM was correct for 97.3% of the samples and PLSDA was correct for 99% of the samples. The combination of NIR microscopy and PCR benefits from the advantages, and overcomes the limitations, of both methods. The combination of the PCR with the NIR microscopy technique can overcome the interference with DNA present in some allowed animal proteins in ruminant nutrition (for example, milk) or other animal products (for example, fat, blood).

Near infrared microscopy analysis on the sediment fraction

De la Haba *et al*. investigated the ability of the NIR microscopy method to discriminate bone particles of fish origin and those of terrestrial animal origin in the sediment fraction.[60] A total of 2010 spectra from sediment particles were collected, including 510 fish meal particle spectra. The spectra were split into two sub-sets, a calibration set and a validation set. The calibration set included 1380 particle spectra with 420 fish meal particle spectra and the validation set included 630 particle spectra with 90 fish meal particle spectra. Two discriminant equations [fish vs non-fish and mammalian MBM (MMBM) vs non-MMBM] were established using the SVM algorithm.[61] For the calibration set, the correct classification success rate was 100%. For the leave-one-out cross-validation and for the validation set, success rates of 95% and 95.5% were obtained, respectively. The SVM models were applied to other samples, such as pure fish meal containing 0.1% MMBM, compound feed containing 0.1% fish meal and 2% MMBM and compound feed containing 0.1% MMBM and 1% fish meal. In all cases, the SVM equation was able to detect the presence of fish meal and MMBM. For three validation samples, the numbers of analysed particles were 344, 332 and 493, respectively, and the number of detected mammalian MBM particles were 152, 5 and 30, respectively. Compared with the MMBM theoretical content, the percentage of MMBM particles detected was too high.

As previously stated, species identification by NIR microscopy is feasible, especially for terrestrial vs fishmeal, the premise for this conclusion being that an authentic species-specific MIRM spectral database has been established.

Obtaining authentic samples of different origin is an important and difficult issue. As explained, the confirmation of species of the samples to include has to be done by complementary techniques, such as PCR. Most papers focus on the

discrimination of terrestrial from fishmeal. The discrimination power of NIR microscopy for the identification of PAPs at higher taxonomic levels (for example, mammalian vs non-mammalian, or ruminant vs non-ruminant) should be demonstrated or validated by further research.

Quantification of processed animal proteins

Only feasibility studies on the application of NIR microscopy to quantify PAPs have been reported over the last few years. NIR microscopy applications in quantification of PAPs are summarised in Table 4.

Analysis on the raw fraction

The first attempt to use NIR microscopy to quantify the presence of PAPs was made by Piraux and Dardenne.[52] These authors applied a regression model between the proportion of MBM in compound feed and the area proportion of MBM particles. Image analysis was used to measure the area proportion of MBM particles. Non-adulterated compound feeds and MBM mixed in at weight proportions of 0%, 2%, 4%, 6%, 8% and 10% were used to build the calibration model. Three independent validation samples containing 2%, 6% and 6% MBM were prepared by another laboratory and used to test the model constructed. Using NIR microscopy, the estimated results were 2.02%, 4.57% and 3.36%, respectively. The results, although they were not sufficiently accurate for quantitative control, were promising. In this study, quantification based on the area of PAPs, does not take into account the difference in density of the different types of particles. Further developments or new methods are required to improve the accuracy of quantification.

Baeten *et al*. conducted a large study to assess the potential of NIR microscopy for the quantification of PAPs.[51] In this study, the performance of the method in quantifying MBM in fish meal was tested. To do this, the raw fraction of several fish meals was analysed and the quantification results were obtained by calculating the percentage of particles classified in the animal group over the total number of particles classified in one of the groups. For 10 fish meal samples spiked with MBM at various levels (3%, 6% and 9%), there was an R^2 of 0.65 between the reference values and the percentage estimated by NIR microscopy. The results showed the potential of the method for the quantification of MBM in fishmeal. However, the accuracy of the method needs to be drastically improved.

Pérez-Marín *et al*. developed a method using a library of animal meal by-products only for quantifying terrestrial MBM in fish meal.[57] The method was based on a two-stage strategy. In the first stage, animal particles were indentified using a global or local distance measure. In the second stage, kNN was used to discriminate terrestrial and fish particles. The proportion of terrestrial MBM in fish meal was calculated by the ratio of the number of terrestrial particles to the total number of particles. Then 20 experimental mixtures of fish meal spiked with various bovine meals (2.5–16%) were used to validate the method. About 200 particles per sample were analysed by NIR microscopy. The percentage of particles classified as terrestrial showed broad agreement with the true sample composition, but the accuracy was too low to be used as a criterion for quantification (an R^2 of 0.42 between the reference values and the percentage estimated by NIR microscopy).

More recently, Abbas *et al*. proposed the key parameters needed to develop a quantitative NIR microscopy approach in order to meet the new European legislation requirements.[62] The proposed parameters included a no-sample preparation, an optimisation of parameters, the use of both the gross (>250 µm) and fine (<250 µm) fractions of samples, the use of the transmission mode to analyse the fine raw fraction and the reflection mode for the gross raw fraction. Further work is needed to develop an accurate quantification method based on these recommendations.

All these results show that the proposed protocols based on the NIR microscopy technique for quantification can be used, at best, as a semi-quantitative method. For accurate quantification, however, there is still much work to do.

Analysis on the sediment fraction

The possibility of quantifying PAPs in a compound feed by analysing the sediment fraction has been also investigated.[51,54] In order to obtain the sediment fraction, the protocol used in EC Regulation 152/2009, and also valid for OM, was used. Some 48 validation samples were prepared using 24 compound feeds (destined for cattle, goats, poultry, pigs and rabbits) spiked at different percentages (0.5% to 8%, w/w in 0.5% intervals) of eight PAPs from different sources in term of species origin and rendering process. The estimated percentage of animal ingredients in spiked compound feeds was calculated as follows: animal ingredients in the sample estimated by NIR microscopy (%) = [weight bones in the sample estimated by NIR microscopy (g)/% bones in the animal feed ingredient used to spike the sample (= *f* factor)].

For 48 samples spiked with eight PAPs, the coefficient of determination (R^2) between reference and estimated values was about 0.57. The R^2 for individual PAPs ranged from 0.64 to 0.97. These values show that the quantification of PAPs using the NIR microscopy protocol based on the sediment fraction is highly dependent on the PAPs source, as observed in the quantification protocol based on OM.[51] It should be stressed that usually the percentage of bones in the PAPs (i.e. *f* factor) is not known for unknown contamination samples.

In order to quantify by NIR microscopy the banned PAPs in the sediment fraction of feedstuffs, two protocols to obtain the sediment were tested. The first uses only tetrachloroethylene to prepare a sediment fraction that has a density higher than 1.62 g mL^{-1}.

The second method (also called the French method) uses two solvents, tetrachloroethylene and tetrabromoethane, to prepare a sediment fraction with a density between 1.62 and 2.2 units. Fifteen samples adulterated with PAPs at levels of 0%, 0.05%, 0.1%, 0.5% and 1% were used to study the quantification. The percentage of animal ingredients in the feeds, calculated by NIR microscopy, ranged from 0.186% to 1.508%

Table 4. Quantification of processed animal proteins by near infrared microscopy.

Study purpose	Training set	Validation set	Model; Method	Performance	Raw/ sediment fraction	Importance and/or weaknesses	Ref.
To demonstrate the potential of NIR microscopy for quantifying MBM contents in compound feeds	780 particles from 56 allowed raw materials; 379 particles from 43 forbidden animal meal products	10 compound feed adulterated with different MBM at levels of 0%, 2%. 4%, 6%, 8% and 10%; three compound feed adulterated with 2%, 6% and 6% MBM.	Regression model between the proportion of MBM in compound feed and the area proportion of MBM particles	$y = 0.61x$, $R^2 = 0.86$ (calibration) 2.02%, 4.57% and 3.36% for 2%, 6% and 6%, respectively. (validation)	Raw	Further analysis are required to improve the accuracy; Quantification based on the area, does not consider the different density of different particles	52
To quantify MBM content in fishmeal	NA	10 fish meal samples spiked with MBM at various levels (3%, 6% and 9%)	Calculation of the percentage of particles classified in the animal group over the total number of particles classified	$R^2 = 0.65$ (validation)	Raw	This method of quantification can spot the trends of MBM contents in fishmeal, but the accuracy need to be improved; Quantitative analysis was performed by calculating ratios of numbers of particles. The sizes and densities of particles from different ingredients are not considered	51
To quantify terrestrial MBM content in fishmeal	11,727 spectra of pure terrestrial meals and 5843 of fishmeal	20 experimental mixtures of fish meal spiked with various bovine meals at various levels (2.0–16%)	Ratio of the number of terrestrial particles to the total number of particles	$R^2 = 0.42$ (validation)	Raw	The accuracy of identifying fish meal and terrestrial MBM need to be improved; Quantitative analysis was performed by calculating ratios of numbers of particles. The sizes and densities of particles from different ingredients are not considered	57

Table 4 (continued). Quantification of processed animal proteins by near infrared microscopy.

Study purpose	Training set	Validation set	Model; Method	Performance	Raw/ sediment fraction	Importance and/or weaknesses	Ref.
To quantify MBM contents in compound feeds	NA	48 validation samples were prepared using 24 compound feeds (destined for cattle, goats, poultry, pigs and rabbits) spiked at different percentages (0.5–8%, w/w in 0.5% intervals) of eight PAPs from different sources in term of species origin and rendering process	Animal ingredients in the sample estimated by NIR microscopy (%) = [weight bones in the sample estimated by NIR microscopy (g)/% bones in the animal feed ingredient used to spike the sample (= *f* factor)]	$R^2 = 0.57$ (validation)	Sediment	The quantification of PAPs using the NIR microscopy protocol based on the sediment fraction is highly dependent on the PAPs source; The f factor (i.e. the percentage of bones in the PAPs) is difficult to obtain for unknown samples	51, 54

NA, not available.

for the one-solvent method and from 0.089% to 1.018% for the two-solvent method. High R^2 values of 0.93 and 0.99 between the reference values and the percentage estimated by NIR microscopy were obtained for the one-solvent and two-solvent methods, respectively. Compared with the true PAP content, the two-solvent method gave a more precise result than the one-solvent method,[54] probably due to the fact that, in the two-solvent method, the sediment percentage was highly correlated to the percentage of MBM ($R^2 = 0.97$). However, when the two-solvent method was used to prepare the sediment fraction for OM analysis, bone fragments might be overlooked and a higher number of false negative results might appear.[15]

Compared with quantification based on the sediment fraction, quantification based on the raw fraction is more promising. However, the published papers do not consider the size and the density of the particles. These variables should be taken into account in further studies.

Comparison of the near infrared microscopy and optical microscopy methods

Compared with OM, the NIR microscopy method does not require an experienced analyst to conduct the analyses. When using NIR microscopy, the subjective judgment of the analyst is replaced by a particle-specific spectrum that can be identified by calibration equations or decision rules. Baeten *et al*. used 17 samples to compare the results of NIR microscopy with those from OM.[54] Table 5 summarises the results of these analyses. All the samples were shown to be positive. For the bone weight in the sediment obtained by the OM and NIR microscopy methods, the correlative analyses were conducted and the determination coefficient R^2 was 0.87. In addition, the Student *t*-test ($\alpha = 0.05$) showed that there was no significant difference between the two results. The NIR microscopy method does not underestimate or overestimate bone content in the sediment fraction. These results demonstrate that the NIR microscopy method can give results equivalent to those obtained by OM, the official European Union method. Compared to OM, the NIR microscopy method does not depend on the skills of the analyst and the whole process can be automated.

Further developments in the near infrared microscopy methodology

Combining near infrared microscopy and other analytical technologies

As the NIR microscopy methodology is non-destructive, the particles classified as being of animal origin can be selected and subjected to another analytical procedure. An analytical procedure combining NIR microscopy and PCR protocols was recently proposed.[59,63] This involved spreading animal PAP particles on a spectralon plate and analysing them by NIR microscopy to identify their plant or animal origin and to give a rough idea of

Table 5. Results from near infrared microscopy and optical microscopy analyses.[55]

Theoretical MBM (%)	Results from NIR microscopy analysis (%)	Results from OM analysis (%)
0.5	0.38	0.51
1	0.75	1.10
1.5	1.48	1.46
2	4.03	4.56
2.5	3.01	3.62
3	5.99	4.06
3.5	2.63	3.62
4	6.79	5.60
4	4.79	5.50
4.5	4.03	3.32
5	5.55	3.49
5.5	4.41	3.59
6	2.96	4.84
6.5	5.40	8.99
7	13.84	10.53
7.5	7.25	7.55
8	10.29	8.28

species origin. The particles classified as animal particles were then recovered and put individually into wells of the PCR plate. Real-time PCR protocols adapted for single-particle analysis and targeting short mitochondrial DNA fragments identified the species origin of these particles. The combination of NIR microscopy and PCR in a single procedure is an elegant solution that benefits from the advantages, and overcomes the limitations, of both methods. Furthermore, this combination can provide a new strategy to establish authentic species-specific NIR microscopy spectral databases. It is a time-consuming procedure, however; the limitation of this combination of methods is essentially the number of particles that need to be manually analysed by NIR microscopy and isolated prior to analysis of their DNA.

Mapping and imaging

For analysing samples spiked with PAPs at levels as low as 0.1%, at least 3000 particles should be scanned. This would take several days to scan, which is unrealistic. Using a mapping system coupled with NIR microscopy or new techniques such as hyperspectral imaging make it possible to overcome this limitation.[61,64,65,66] By using the NIR microscopy mapping option, a quantity of the feed sample is mapped by scanning an area that is divided into equally spaced points in both *X* and *Y* directions, based on a scan interval adapted to the size of the fraction (<250 µm). Spectra are automatically collected at these points. Animal origin detection can then be done, as explained earlier, using classification equations or decision rules. Quantification can be also done, as explained in the section "Quantification of processed animal proteins".

Hyperspectral images are three-dimensional datasets containing light intensity measurements where two dimensions (*x* and *y*) represent spatial distances and a third dimension (λ) represents spectral variation such as wavelength. They can be interpreted as stacks of, typically, hundreds of two-dimensional spatial images at different wavelengths, or tens of thousands of spectra aligned in rows and columns. Three instrumentation approaches are used to obtain hyperspectral images, known as point, line or plane scans depending on the orientation of the scanning dimension relative to the two-dimensional spatial sample axes. NIR hyperspectral imaging systems have been proposed for successfully detecting MBM in feed in combination with decision rules or chemometric discriminant equations.[49,61,64,65] The recent success of NIR hyperspectral imaging can be attributed to a combination of various factors: (1) a non-destructive method, (2) digitally tuneable infrared optical filters and (3) a drastic increase in both the speed and capacity of laboratory computing platforms. A framework for developing and validating an NIR hyperspectral imaging method as a standard protocol has been proposed.[66] This involves using various criteria and tests to assess LOD, repeatability and risk of cross-contamination and to validate the NIR hyperspectral imaging method for detecting PAPs in compound feed in line with ISO 17025.

Conclusions

The NIR microscopy method combines the analytical advantages of microscopy and spectroscopy techniques. The

principle of NIR microscopy analysis is similar to the OM method for detecting MBM in feed. Current research shows that NIR microscopy is an objective, sensitive and highly selective technique for detecting MBM in compound feed. Compared with the reference method (i.e. OM), the main advantages of NIR microscopy are that it is non-destructive, can be automated and does not require experienced analysts.

The evolution of the NIR microscopy methodology has been summarised in this paper, including detection based on discriminant models and decision rules, species identification, quantification, comparison with OM, combination with other techniques such as PCR, transferability and further developments in the area. Since 2006, NIR microscopy has been used for routine analysis within the framework of the activities of the Community Reference Laboratory for Animal Proteins in Feedstuffs (CRL-AP, www.crl.cra.wallonie.be). These analyses are performed in line with ISO 17025.

NIR microscopy is an interesting alternative technique for the detection of PAPs. However, the detection greatly depends on the degree of homogeneity of the sample and on the number of particles analysed. When the content of animal ingredients in an adulterated sample is too low, a large number of particles should be analysed. Increasing the number of particles analysed by unit of time is a critical issue for the development of NIR microscopy for the detection of PAPs in feeds. The use of NIR microscopy coupled with a mapping system or the use of hyperspectral imaging could be promising solutions.

The European extended ban (preventing cannibalism) will be lifted if reliable analytical methods for the species specific identification of PAPs in feed become available. As noted in this paper, NIR microscopy has shown potential for the species classification of PAPs of various origins, especially the discrimination of terrestrial and fish meal. Further research is needed, however, with regard to the discrimination of higher taxonomic levels, such as poultry, mammals and even ruminants and pigs. Efforts also have to be made to accelerate the combined NIR microscopy/PCR method and establish the species-specific NIR microscopy spectral database in order to be able to test a large number of species simultaneously. Combining the NIR microscopy method with other protocols that allow species-specific detection (for example, analysis of species-specific proteins by mass spectrometry) needs to be tested and encouraged.

The potential of the NIR microscopy method to quantify MBM concentration in feed could not yet support the eventual introduction of a tolerance level in the feed ban. Currently, for the raw fraction, the quantitative analysis was performed by simply calculating ratios of numbers of particles. The sizes and densities of particles from different ingredients are not the same and this could be the main reason for the difference between theoretical value and NIR microscopy estimated value. For the sediment fraction, the *f* factor (i.e. the percentage of bones in the PAPs) is an additional constraint in setting up a quantitative protocol because it is difficult to obtain it for unknown samples. Compared to the quantification protocol based on the analysis sediment fraction, the protocol based on the raw fraction is more promising. However, the size and density of particles have not been considered in the published studies. Further work is needed to develop an accurate quantification method based on NIR microscopy.

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