Key parameters for the development of a NIR Microscopic method for the quantification of processed by-products of animal origin in compound feedingstuffs

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Abstract

The aim of this work is to show new advances in the analytical methods developed in the frame of the ban of processed animal by-products in compound feed that is currently applied within the European Union. With this aim, studies to develop a quantitative near infrared microscopy (NIRM) approach have been undertaken in order to fulfil the new requirements of the European legislation. The capacities of the NIRM method have been improved; no sample preparation is requires and the acquisition parameters are optimized. Both the gross and the fine fractions of the samples are considered; the transmission mode was chosen to analyze the fine raw fraction while the gross raw fraction was analyzed in the reflexion mode. Parameters for reflexion analyses were already fixed in previous studies while those of transmission mode have been determined in the present study. Because the diameter of the particles is too small, which makes it difficult to mark particles; spectra were collected using the mapping technique. Quantitative analyses have been carried out for different percentages of adulteration (0.5, 1, 2 and 5%). The established protocol with the key parameters proposed has to be considered for the development of an accurate method of quantification.

Keywords: NIR microscopy, transmission, mapping, animal feed, contamination, quantification.

Introduction

Epidemiological studies have associated bovine spongiform encephalopathy (BSE) to animal contaminated feed as a major BSE transmission route in cattle. Contaminated ruminant protein enters the feed chain mainly in the form of meat and bone meals (MBM). Forbidden MBM can also be included accidentally or can result from a cross-contamination at feed mills during transport, storage or at the farm level[1].The European Commission Decision of 94/381/EC[2] prohibited the use of proteins derived from all mammalian tissues in feedstuffs destined for ruminants. Later, Council Decision 2000/766/EC (2000)[3] prohibited processed animal proteins from rations destined for farmed animals that are used for food production. Derogations have been introduced for various processed animal by-products such as hydrolyzed proteins, fish meal, dicalcium phosphate and gelatines for animals other than ruminants. All this has been transferred into a permanent ban by Commission Regulation (EC) No 1234/2003[4]. Policies concerning raw materials in feed formulation require developing and continuously adapting the methods used for the detection of illicit ingredients. Optical microscopy (OM) is the official method for the detection of MBM in compound feed[5,]. But, the limitations of this method have led to the development of alternative methods based on molecular biology (e.g. Polymerase Chain Reaction "PCR" and Immunoassays)[6,7,8,9,10], and spectroscopy (e.g. Near Infrared Spectroscopy - NIRS)[11,12,13] to improve the detection and the identification of specific materials of animal origin[7,8,9,10]. In order to enhance performance of spectroscopic methods for the detection of animal proteins, recent developments have led to the use of near infrared microscopy method (NIRM)[14,15]. The advantage of NIRM is that it combines the optical microscopy and spectroscopy benefits. Near infrared hyperspectral imaging has been also recently proposed as an alternative method[16]. This technique permits to obtain, at the same time, spatial and spectral information characterising the samples. Chemometric tools like Partial Least Squares Discriminant Analysis (PLS-DA), Artificial Neural Networks (ANN), and Support Vector Machines (SVM) have been successfully developed and applied to NIR spectra in order to distinguish the particles of animal origin from the others[17].

The microscopic methods (NIR microscopy and NIR hyperspectral imaging) have been revealed to be efficient for the species specific detection of animal particles[12,13,14,18,19]. Proposed methodologies have been developed to assure, with an acceptable level of confidence (95%), the detection of at least one animal particle when a feed sample is adulterated at a level of 0.1%[20]. Both methods are running under accreditation ISO 17025 since 2005 at the Walloon Agricultural Research Centre (CRA-W).

Nowadays, the knowledge of the percentage of animal protein in feedstuff is of main importance for a proper evaluation of the contamination level. Providing an adequate way of quantification

becomes a major challenge for scientists and policy makers. In fact, quantification of traces - caused by unavoidable cross-contamination - of specific processed animal proteins (PAPs) such as fish meal in feedingstuffs for ruminants is a crucial factor to facilitate the use of these PAPs in animal nutrition of non-ruminants.

The quantification by optical microscopy has proven to be difficult to achieve[21]; quantification is performed on the sediment fraction and the protocol specifies that the quantification is only possible if there are particles of bones/scales in the constituent of animal origin.

The quantification method by optical microscopy is based on the Commission Regulation 152/2009 [5]. The formula used for the calculation of the estimated value (in %) of constituents of animal origin is the following:

$$\% = \frac{S2 \times d}{W \times f} \times 100$$

Where *S2* is the weight of the dry sediment (in g), *W* is the weight of the sample material for the sedimentation (fixed to 10g), *d* is the correction factor for the measured portion of fish bones in the sediment (in %), *f* is the correction factor for the proportion of bones in the constituents of animal origin in the sample examined. In the case of the quantification of terrestrial MBM the factor *d* is replaced by "*c*", which is the measured portion of terrestrial bones in the sediment (in %)**.**The accuracy and reproducibility of the method are limited by the fact that the proportion of bones in the constituents of animal origin *f* is rarely known and has to be arbitrarily fixed. Moreover, the performance and reproducibility of the method depend mainly on the skills of the operator, which influence mainly the factor *d* (or *c*) [19,22,23] Therefore, there is strong need to develop alternative strategies to overcome the limitations of this method.

The objective of the present work is to demonstrate the ability of NIRM to quantify processed by-products of animal origin in compound feedingstuffs and to give some key parameters to estimate the contamination percentage of pure fish meal in animal feed formulation. The fitness for purpose of the developed NIRM method is demonstrated by analysing compound feed samples fortified with fish meal at various concentrations.

Materials and methods

Procedure

The compound is first ground to a size of 1 or 2 mm. When applying classical or near infrared microscopy, the compound is measured as it is (raw fraction) or after concentration (sediment fraction). The sediment is obtained through a sedimentation process in tetrachloroethylene that will gather particles above a defined density. Raw and sediment materials are passed through a sieve of 250 µm diameter and the obtained fractions are then examined. The gross fraction contains particles

with diameter in the range 250 - 1000 µm and the fine fraction includes particles in the 0 - 250 µm diameter range. In the analysis of the raw fraction, detection is based on the presence of different kinds of particles (e.g. bone or muscle) of animal origin. In the case of the sediment fraction, detection is based on the presence of specific animal particles (e.g. bone).

In the present work, only the raw fraction, called in the present paper whole sample, was studied and analysed by NIRM method.

Instrumentation

An auto image microscope connected to a Fourier transform near infrared spectrometer (PerkinElmer) was used. The instrument permits to collect spectra from small surface (10 µm x 10 µm). The microscope includes a camera and a viewing system to magnify the visible-light image of the sample under observation. Spectra were collected between 9000 and 4000 cm^{-1} with a resolution of 8 cm^{-1} and a total of 10 co-added scan. The sample spectra are the result of a ratio between the raw spectra and the background. The background was made on a spectralon in the case of reflexion analyses and on a slide in the case of transmission mode.

The selection of the method to analyze each fraction depends on its diameter.

Gross fraction: about 300 particles were spread on the sample holder (i.e. spectralon). Particles were marked manually then spectra were collected in the reflexion mode as already developed in the literature[24].. This procedure was repeated until 900 particles of each sample were analysed.

Fine fraction: Trials have been performed to analyze the fine fraction (< 250 µm). The best way to analyze this fraction is the transmission mode. The beam light is condensed to an appropriate size for the microscopic scale then focused at the sample position. This way of spectra collection is suitable for fine particles for their small diameter. About 1024 spectra were collected for each sample. This option has not been applied yet in the literature and has been fully developed and is presented in this paper. The mapping option of the microscope was applied. A quantity of the feed sample is mapped by scanning an area, which is divided into equally spaced points in both X and Y directions according to a scan interval adapted to the size of the fraction (< 250 µm). Spectra are automatically collected at these points. The quantification is based on the number of spectra identified as specific for fishmeal related to the total number of spectra by taking into account some parameters like the density of particles and the contribution of each fraction in the composition of the analysed sample. For the identification of the spectra measured from the both fractions we applied decision criteria already defined in the framework of the accreditation of the NIRM method according to ISO 17025. The rules developed by the CRA-W are based on the visual observation of the spectrum in order to assess the animal origin[25].

Treatments

In order to calculate the total percentage of adulteration (equation 4) expressed as percentage mass portion, several factors were considered: the theoretical percentage, the percentage obtained with the reflexion mode (equation 1), the percentage obtained with the transmission mode (equation 2), and the percentage of each fraction (equation 3) in the sample. The density of the animal protein has been determined by calculating the ratio between the weight (mg) of 100 pure animal particles and the weight (mg) of 100 vegetal particles; the value approximates 1.39. Percentages were then calculated as follows:

$$\%\,reflexion = \left(\frac{number\ of\ spectra\ of\ animal\ particles}{number\ of\ analyzed\ spectra} * 1.39\right) \quad (1)$$

$$\%\,transmission = \left(\frac{number\ of\ spectra\ of\ animal\ particles}{number\ of\ correct\ analyzed\ spectra} * 1.39\right) \quad (2)$$

Correct analyzed spectra: the use of mapping option in the transmission mode leads to collect spectra of particles but also of the slide in some cases or spectra collected at the contour of the particles. So, noisy spectra were withdrawn from the calculation.

$$\%\,(gross\ or\ fine\ fraction) = \frac{weight\ of\ the\ gross\ (or\ fine)\ fraction}{Total\ weight\ of\ the\ sample} \quad (3)$$

$$\%\,total = \frac{\%transmission \cdot weight(fine\ fraction) + \%\,reflexion \cdot weight(gross\ fraction)}{weight(fine\ fraction) + weight\,(gross\ fraction)} \quad (4)$$

Samples

Spiked samples obtained by mixing matrices of compound feed with MBM or pure fish meal have been intensively shaken using a whisk in order to be well homogenized.

Three different sets of samples were used to perform this study, each of them was dedicated to one specific aim (set 1, set 2, and set 3):

Sample set 1 is composed of 5 samples of compound feed (blank material) ground at 2 mm and 2 PAP samples (pure fish and pure bovine processed animal proteins) ground at 1 mm.

Samples were sieved at different diameters as described in Table 1 in order to consider the corresponding particle size distribution of compound feed, meat and bone meal and fish meal, respectively.

Sample set 2 was comprised of 15 feedingstuff samples adulterated at different percentages 0.25, 0.5, 1, and 1.5 % of pure fish meal. The objective of the preparation of sample set 2 (Table 2) was to

investigate the relation between the total number of particles analysed representing the required sample quantity, the number of particles detected as fish meal and the calculated percentage mass portion of fish meal in the compound feed samples.

Sample set 3 as shown in Table 5 was used to apply the developed method of quantification on 14 compound feed samples fortified with fishmeal at various concentrations. Samples consisted of a feed formulation intended for cattle or pig, fortified with a mix of different pure fish meal samples (from Faroe, Peru, Panama, France and Iceland , blue whiting fish meal steam dried and salmon mixed to white fishmeal produced on indirect steam drier), at 0.5, 1, 2, and 5% .

Results and discussion

1. Parameters to develop a NIRM quantification method

The development of a quantitative method implies to improve microscope capacities and to fulfil additional requirements; a) to avoid an intermediary step of preparation of the sample, b) to determine a representative sampling considering the particle size distribution in the different fractions, c) to define a representative portion of the sample expressed as total number of particles and d) to optimize the acquisition spectra parameters.

a) Spectroscopic analysis of the whole sample without sedimentation

The best way for the quantification of PAP should not include the intermediary step of sedimentation as described in the official method (EC Regulation 152/2009)[5]. This will avoid the problem due to the unknown factor *f* which is one of the major drawbacks of the protocol. The indicated procedure implies to quantify bones or bones/scales only in the sediment fraction (determination of *c* or *d*). Here we utilise the unique characteristic of the NIRM method that allows the detection of animal particles whatever in the sediment or in the whole sample. Therefore the approach for quantification developed in this study is based exclusively on the whole sample.

b) Determination of a representative sampling

For the quantitative purpose it is mandatory to analyze the particles from the gross and the fine fraction obtained from the whole sample. This is essential because, adulterant material is not necessarily grounded at the same size as it is for the rest of the sample. We used sample set 1 to elaborate on this aspect. As shown in Table 1 the percentage of the gross fraction of the compound feeds (samples “a” to “e”) is about 79.8 - 93.5 % while it is about 49.4 - 54.5 % in the case of PAP (samples “f” and “g”). That means that when a matrix is mixed with a PAP presented in this table, an important bias will be introduced when only one fraction is taken into account. Indeed, the analysis

of only the gross fraction leads to an underestimation of the real percentage. Similarly, if only the fine fraction is analyzed, an overestimation of the real percentage will be obtained. In order to illustrate the effect of conducting the analysis on exclusively one fraction, we use the theoretical example that the compound feed sample "a" of Table 1 contains fish meal (sample "g") at a concentration of 1 %. When exclusively analysing the fine fraction, the estimated fish meal concentration of the whole sample would be about 5.8%, whereas the analysis of exclusively the gross fraction would result in an estimate for the fish meal of about 0.54 %. In both cases a significant bias would be introduced into the measurement.

Table 1

Both the fine and gross fractions are then considered in this study to assure a good accuracy.

c) Definition of the representative portion of the sample

Another essential issue is the fact that the number of particles that will be analyzed should be a representative portion of the sample under investigation. Generally, the quantity of the samples put on the sample holder is less than 100 mg, which represent about 300 - 400 particles (if we consider the gross fraction). The weight of one particle has been determined: one particle from the 1 mm – 500 µm range has a weight of about 0.15 mg and one particle from the 500 µm – 250 µm range has a weight of about 0.03 mg. This means that the average weight for a particle from the 1 mm – 250 µm range is about 0.09 mg. In previous studies, it has been demonstrated that the detection of at least one particle in a sample spiked at 0.1%, requires the analysis of 3000 particles[19,26,27] that corresponds to about 270 mg. Moreover, it has been determined that animal particles have a weight higher than 38% regarding plant particles. In order to evaluate the representativity of the analysis of 3000 particles for a quantitative aim, samples of the set 2 have been analyzed.

Table 2

Table 2 shows the theoretical percentage of contamination for each sample, the experimental value after counting the spectra of detected animal particles then considering the correction for the fraction influence and the influence of the density. The fraction influence equal to 1.81 is calculated on the basis of the ratio between the percentage of vegetal particles having a diameter superior to 250 µm and the percentage of pure fish particles having a diameter superior to 250 µm equal to 90 and 50 % respectively. Percentages were determined experimentally. Finally, the density of the animal particles, equal to 1.39, is taken into account to calculate the experimental percentage of contamination.

Theoretical and experimental percentages of contamination are quite close for percentages of adulteration equal to 1 and 1.5 %.

Results indicate that in the case of a sample spiked at 1 % or 1.5 %, between 3 and 10 particles are identified from 1000 particles analyzed.

d) Optimization of the acquisition spectra parameters

Analyses have to be made on both the gross and the fine fractions. The protocols used until now were focused on the particles higher than 250 µm (gross fraction). Analyses were made in reflexion mode and the parameters of spectra acquisition have already been fixed[21]; being the number of scans of the background (i.e.spectralon) equal to 100, the number of scans of sample spectra equal to 10, and the aperture size 50 µm x 50 µm. Particles are spread on a spectralon and marked individually in order to collect their NIR spectra.

For the transmission mode analysing the fine fraction, the particles were spread on a slide on which the background was collected. In this case the number of scans was increased to 150 in order to insure a good quality of spectra. Because the particle diameter in the fine fraction is very small, marking has been revealed tedious and time-consuming. It was then decided to investigate the application of a mapping technique. The fraction was mapped by scanning the surface according to an interval which has to be defined. Before that, the aperture size in transmission mode has to be adapted to the size of the particles (< 250 µm).

d.1) Optimization of the aperture

The fine fraction particles were spread out into a mono-layer over a slide (usually used in optical microscopy) covering an area of 1 cm^2. Five particles (in the 0 – 250 µm range) from one sample of the set 3 were marked individually and analyses were carried out. Different aperture sizes were tested: (10 µm x 10 µm), (15 µm x 15 µm), (20 µm x 20 µm), (25 µm x 25 µm), (40 µm x 40 µm), and (50 µm x 50 µm). The results obtained are presented on the **Table 3**.

Table 3

The determination of an appropriate aperture size is important to assure the collection of the spectrum of the target particle. Indeed, a too large aperture increases the risk to cover more than one particle while a too small aperture size leads to bad quality spectra with a weak intensity. It is thus necessary to find a good compromise between these two values.

Two criteria defining the quality of the spectra were considered: the signal to noise ratio, which defines the quality of the spectra, and the spectral intensity, which depends on the quantity of the received energy **(Figure 1)**.

Figure 1

In Figure 1, spectra of particles collected at different apertures are represented. The quality of the spectra increases according to the size of the aperture but on the basis of criteria defined above the retained value of the aperture size is (25 µm x 25 µm). As shown in Table 3, at this aperture the energy level is equal to 2667 and the intensity of the spectrum at 1940 nm is 0.22. In fact, no significant differences in the quality of collected spectra have been observed with the spectra obtained with greater sizes of the aperture; (40 µm x 40 µm), and (50 µm x 50 µm).

d.2) Optimization of the scan interval

This parameter depends on the size of the particle. It should be neither too high leading to miss the scan of one particle nor too small risking to point several times the same particle. Two slides were examined over which the fine particles of one sample adulterated at 1% of PAP were spread out into a mono-layer over a square of 1 cm on side. The slides were analyzed by the mapping technique. Various intervals (175 - 200 - 225 - 250 µm) were tested and then results are compared to the real percentage of adulteration.

Results presented on the **table 4** show that there are no significant differences when using different intervals. No interval permits to approach the theoretical percentage. The scan interval equal to 250 µm was then selected because it allows faster analyses.

Table 4

2. *application of the optimized method on samples at different percentages of adulteration*

In order to estimate the total percentage of adulteration of one sample, several factors were considered (equation 4): the theoretical percentage, the percentage obtained with the reflexion mode (equation 1), the percentage obtained with the transmission or reflexion mode (equation 2), and the percentage of each fraction (equation 3).

Analyses were made on 14 samples (sample set 3) adulterated at different percentages of fish meal (0.5, 1, 2, and 5 %). Experimental percentages obtained with the reflexion, transmission mode and the total one as well as percentages of the gross and fine fraction of each sample are reported in **table 5**.

Table 5

The data indicate that for the low percentages of contamination as for 0.5 %, the reflexion mode permits to give results closer to the theoretical value (0.28 % – 0.85 %). For the other percentages (1, 2, and 5 %), results show an over or under estimation depending on the sample and on the mode of analysis (reflexion or transmission). One sample S042 gives a very high value of adulteration when the fine fraction is analysed. No explanation could be given.

From the table 5, it can be shown that only if *both* fractions are analysed, a bias in the measurement could be avoided. However, this effect can only be demonstrated when the particle size distribution of the feed and of the fish meal is very different – that means samples S013, S014 and S039 (assuming that for fishmeal 50.4 % is in the fine and 49.4 % is in the gross fraction). In fact, for samples S013 and S014 contaminated at 1 % level; 49.4 % of pure fish (> 250 µm) are diluted in 79.69 or 76.84 % of the sample (gross fraction) while the 50.4 % of pure fish (< 250 µm) are diluted in 23.31 and 23.16 % of the sample (fine fraction) which leads to an experimental percentage of adulteration superior when analysing only the fine fraction (1.85 %, 2.07 %) than it is when analysing only the gross fraction (1.05 %, 0.58%). The same remark can be given when studying the sample S039 adulterated by 2 % of pure fish. Oppositely to S013 and S014, this sample is mostly composed of the fine fraction. 50.4 % of pure fish (< 250 µm) are diluted in 79.13 % of the sample (fine fraction) while 49.4 % of pure fish (> 250 µm) are diluted in 20.87 % of the sample (gross fraction) giving an experimental percentage of adulteration superior when analysing only the gross fraction (11.51 %) than it is when analysing only the fine fraction (4.33%). On the basis on the results obtained with these three samples it is easy to see what happens, when only the fine or gross fraction is analysed. The dilution effect is markedly observed when taking into account only one fraction; both fractions have to be analysed in order to increase the accuracy of the quantification.

Conclusion

The objective of this study was to set some key parameters necessary to develop a method of quantification of the particles of animal origin in feedingstuffs. The quantification method has to be developed on the basis of the whole sample by considering both the gross and the fine fractions. The

gross fraction (>250 µm) has to be analyzed in the reflexion mode while the fine fraction (< 250 µm) has to be analyzed in the transmission mode. Parameters of spectra acquisition in transmission mode were developed and optimized; the number of scans of the background equal to 150, the aperture set to 25 µm x 25 µm with the use of a mapping technique (a scan interval equal to 250 µm).
The experimental percentage of adulteration has to be calculated on the basis of the total of the percentages obtained with the gross and the fine fractions.
Further investigations will be oriented to the use of these recommendations to build an accurate quantification method.

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