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Application of near-infrared microscopy (NIRM) for the detection of meat and bone meals in animal feeds: A tool for food and feed safety

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Abstract

This paper reports on the development and validation of a method for detecting meat and bone meal (MBM) in compound feeds by near-infrared reflectance microscopy (NIRM) as an alternative in food and feed safety. A FT-NIR (Fourier transformer-near-infrared reflectance) instrument attached to a microscope was used to build up a spectral library containing reference feed particles identified as plant or animal origin, from various sources. Spectra were collected directly from particles in the NIR spectrum region (1112–2500 nm). The spectral library sample set was used to develop various discriminant models to classify spectra as MBM or plant material. The best discriminant model was obtained using partial least squares (PLS) discriminant analysis and standard normal variate and detrending (SNVD) and first derivative for spectrum pretreatment; this model had a coefficient of determination of *0.95* and a standard error of cross-validation of *0.133*. The model was externally validated. The results confirmed NIRM as a valuable technique for detection of banned MBM.

Keywords: Near-infrared microscopy (NIRM); Bovine spongiform encephalopathy; Meat and bone meals; Food and feed safety; Spectral discriminant analysis

1. Introduction

The "food crisis" that arose in the European Union following the outbreak of Bovine spongiform encephalopathy (BSE), and the scientific evidence linking this disease with the consumption of feeds containing varying proportions of meat and bone meal (MBM), has led institutions, governments and, above all, consumers to demand strong measures to prevent any recurrence of the disease (2000/766/EC). This crisis has had a direct impact on the feed and food production sectors, underlining the need to strengthen quality controls in these areas.

Recent European legislation governing food safety and traceability has tended to stress that it is the responsibility of the animal feed industry to produce safe feeds (94/381/EC, 96/449/EC, 98/88/EC, 99/129/EC, 2003/126/EC).

The EU Regulation (EC) No. 1774/2002, which came into force in May 2003, regulates the use and treatment of animal by-products obtained after slaughter, and thus revives the debate over the use of processed animal proteins in animal feeds, as first link of production chain, although only for single-stomach animals. At present, the wide-ranging legislation affecting the feed sector requires reliable, accurate, economic and rapid analytical methods capable of fully tracing the complex matrix of foods and feed ingredients and enabling the detection of possible frauds and adulterations. Indeed, the latest EU Directive on the analytical method for the determination of constituents

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of animal origin for the official control of feedingstuffs (2003/126/EC), explicitly recognises the difficulty in developing a method which will guarantee the required sensitivity, specificity and accuracy, and allows the use of techniques other than optical microscopy. The official UE authorised method for detecting MBM in compound feeds is conventional optical microscopy. This technique enables identification of muscle fibres, hair, feathers, bones, cartilage, etc., which provide additional information on the type of meal involved; however, it cannot detect other products of animal origin, such as fat (Gizzi & von Holst, 2002). These limitations, as well as those inherent in the technique itself, are compounded by problems associated with organochlorine waste management, underlining the need for alternative, environment-friendly analytical methods. A number of new technologies have been developed for the authentication and traceability of animal feeds, including macro-NIRS (near-infrared reflectance spectroscopy) (Pérez-Marín, Garrido-Varo, Guerrero-Ginel, & Gómez-Cabrera, 2004; Pérez-Marín, Garrido-Varo, Guerrero-Ginel, & Gómez-Cabrera, 2005), polymerase chain reaction (PCR, Kromar & Rencova, 2003; Chiappini et al., 2005; Myers et al., 2006), and others (Dardenne, Baeten, & Vermeulen, 2004).

Proposed spectroscopic methods for detecting animal meals in compound feeds are based on near-infrared spectroscopy (Baeten & Dardenne, 2001; Pérez-Marín et al., 2005; Garrido-Varo et al., 2005); in particular, near-infrared reflectance microscopy (NIRM) appears to offer most potential for detecting MBM contamination in feedingstuffs, since it can collect spectral information from microscopic particles, and match the results unequivocally against spectral libraries comprising patterns of known composition (Piraux & Dardenne, 2000).

NIRM is an objective, rapid, sensitive and highly-selective technique for controlling meat and bone meals (MBM) in compound feeds. It is based on the collection of spectra from hundreds of particles contained in any feed, using an Fourier transformer-near-infrared reflectance (FT-NIR) instrument attached to a microscope whose highly-developed optical system is designed to focus sufficiently on particles and increase the efficiency of radiation transmission for microspectrum collection; near-infrared spectra can be collected from extremely small particles ($\leqslant$50 μm). The particles present in the feed can be identified by comparison with reference spectral libraries previous developing a discriminant analysis with an appropriate chemometric tool.

Earlier studies by Baeten, Michotte Renier, Sinnaeve, Garrido Varo, and Dardenne (2004) and Baeten et al. (2005), have shown that NIRM is able to detect animal meals in compound feeds, however these proposed methods require tedious sample pretreatment, grinding and extraction with highly-toxic organochlorine solvents. Moreover, each sample is analyzed using conventional microscopy (2003/126/EC) and particles suspicious to be bone fragments using visual detection are characterized finally by their spectral information.

This study sought to develop and validate a method which would enable identification of meat and bone meal, while minimizing sample pretreatment and environmental risk, using NIRM, a powerful spectroscopic tool.

2. Materials and methods

2.1. Spectral library

Numerous raw food and feed ingredients of different origins were used: 2229 spectra from meat and bone meal banned samples were provided by the Sample Bank of the University of Cordoba. Each sample was supplied with an identification card containing all relevant information: origin, percentage of animal species, conditions of processing, and other observations occurred when manufacturing. In order to increase variability, all meat meals included in this study were produced using different types of animal constituents from ruminant and non-ruminant on the basis of rendering subproducts of pork, poultry, sheep, cows and mixture of them. A further 1556 spectra from plant-based feeds (PBF) were used to build up a spectral library of vegetable origin particles. These samples were different raw materials normally used as ingredients on feedstuffs such as barley, maize, soybean, wheat, etc.

2.2. Validation samples

Two different sets were used to validate the analytical method developed:

Set 1: This set comprised 18 compound feeds collected after publication of the total MBM ban (2000/766/EC); and representative of those produced and marketed in Spain after the introduction of that ban. Ten samples were feedstuffs for livestock and therefore free of MBM; while the other eight were pet foods containing between 26% and 35% MBM. Samples came from the Sample Bank of the University of Cordoba.

Set 2: This set comprised six blind samples provided by the Spanish Ministry of Agriculture as part of a joint assay performed during 2004. Five samples were compound feeds containing $\leqslant$1% MBM and four of them containing different blood meal percentages (between 0.02% and 0.3%).

2.3. Sample preparation

The only sample preparation carried out prior to analysis, was grounding at 1 mm sieve, using a cyclone mill (Foss Tecator Cyclotec 1093).

2.4. NIRM analysis

Spectra from different plant and MBM particles were collected using an Auto Image Microscope connected to a

Perkin–Elmer Spectrum One Fourier transformer-near-infrared (FT-NIR) spectrometer in reflectance mode (1112–2500 nm) using a resolution of 8 cm^{-1}. Spectral information was stored as $\log(1/R)$, the final spectrum for each particle being the average of 100 individual spectra.

To build the spectral library, a total of 200 different particles per standard (plant and/or MBM) were scanned. For unknown feedstuff samples, 500 particle spectra were analysed per sample in order to avoid false negatives.

The Auto Image microscope is equipped with a video camera and a viewing system to localise and mark one-to-one the particles of interest to be analyzed. This step is necessary prior to analysis. The protocol used in this study was totally different to that used in optical microscopy. The NIRM analysis was made on ground raw samples (without prior extraction or separation procedures).

After randomly selecting scan points, infrared rays were focused onto particles; the AutoImage collected spectra from small particles (50 μm × 50 μm), and the detector located in the microscope measured the reflected beam. Spectra were obtained as the average of one hundred sub-scans, bearing in mind the ratio of raw spectra to background. A spectralon button provided by the instrument manufacturer was used to collect background. This final spectrum was the fingerprint of the analysed particle.

The software provided by Perkin–Elmer, Spectrum v. 5.01 (Perkin–Elmer, 2002), was used to collect and store spectra. WinISI II software v.1.5 was used for computer operation and spectral data collection analysis (WinISI II, 2000).

2.5. Discriminant analysis

Spectra were exported from the Spectrum software in ASCII format into the WinISI II software v.1.5 for chemometric analysis to perform discriminant analysis using partial least squares (PLS2) (Murray, Aucott, & Pike, 2001). Two files were involved: a file of plant-based feeds (PBF) containing 1556 spectra and a file of meat and bone meals (MBM) containing 2229 spectra. A calibration matrix was set up with zeros and ones against these file names; these are sometimes known as "dummy variables". Calibration was performed by regressing the wavelength data on the groups defined as zero or one; cross-validation was used to test the accuracy of the model at each step. Results are shown as a table giving the number of factors, the number of misclassified samples and the standard error of cross-validation (SECV). The final equation developed was then used to discriminate the unknown samples, assigning them either to the filename for PBF or to the filename for MBM depending on which file had the greater score.

Different chemometric strategies were evaluated, using raw and pre-processed spectra. They were used raw spectra without pre-treatment where $\log(1/R)$ was not scatter corrected and pre-processed spectra applying scatter correction consisted on standard normal variate and detrending (SNVD) of $\log(1/R)$ at each wavelength (Barnes, Dhanoa, & Lister, 1989, 1993) as different options offered by the software (WinISI II, 2000).

Mathematical treatments used were: non-derivative, first derivative and second derivative, using 172 wavelengths and between 8 and 13 PLS factors depending of mathematical treatment used. These are denoted 0,0,1,1; 1,4,4,1 and 2,5,5,1, respectively. The first number denotes the derivative order, while the second number denotes the number of nanometres in the segment used to calculate the derivative. The third and four numbers denote the number of data points over which running average smoothing was conducted.

For discriminant analysis, reference samples in the calibration set were pre-assigned to their specific groups, i.e. PBF or MBM. The discriminant function clustered together as closely as possible the samples in the same group, and perfectly separated one group from the other in the multidimensional space.

For each group, the scores files added up to three; with only two groups, PBF and MBM, a predicted score of around 1.5 in both files meant that assignment could go either way, scores around two meant samples correctly assigned, and lower than one meant mis-assigned.

For developing classification models, was used the criteria *uncertainty factor* and *minimum difference*. The uncertainty factor is related to the value for Student's T statistic for infinite degree of freedom. The uncertainty factor used here was 0.5, which corresponds to Student's T value of 1.76 and a probability of 96%.

The *minimum difference* value is directly related to the Student's T value and the error of cross-validation; the *minimum difference* declines with the T value, and thus with the uncertainty factor.

3. Results and discussion

The NIR spectrum of a particle is identified as its unequivocal spectral fingerprint; thus, a population will be represented by the average spectrum of its specimens. Fig. 1 shows average spectra for the two sample popula-

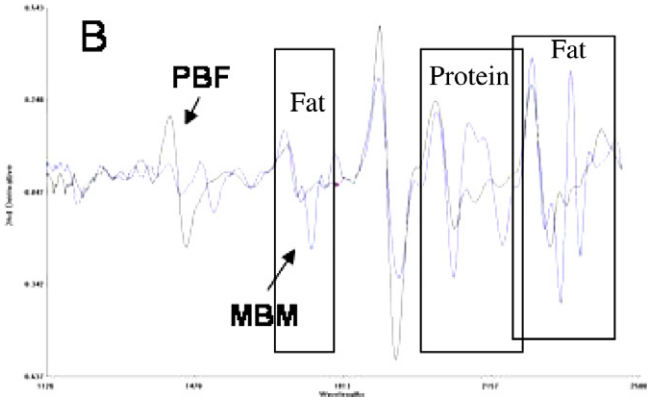

Fig. 1. Near-infrared mean spectrum of MBM and PBF populations applying second derivative. Spectral characteristics.

tions after applying second derivative to the original spectra. With both pretreatments, differences between the two populations were even more marked than, in $\log(1/R)$ mode. The average spectrum for the MBM and PBF populations; the main characteristic bands differentiating the two spectra are clearly visible, and there are considerable differences in the location and shape of the bands, in the regions characteristic of fat absorption (1724–1760 nm and 2306–2346 nm), according Murray et al. (2001) this bands are related with the content in polyunsaturated fatty acids, and protein absorption (2054–2274 nm) (Williams, 2001). Other important bands to remark are: C–H first overtones at 1700 nm. These differences served to confirm the feasibility of developing a prediction model able to discriminate between MBM and PBF in raw materials; they also suggested that the best results might be obtained applying a derivative treatment to the original spectra.

Chemometric models, as mentioned before, were developed using discriminant analysis, applying the PLS2 algorithm (Murray et al., 2001). After application of the center algorithm, all the treatments assayed repeatedly yielded aberrant statistics for two particles belonging to the MBM set. Fig. 2 shows clearly the differences between these two outliers (L114 and L115) and the average spectrum for the MBM population. Both spectra were very noisy, and were thus excluded from the calibration set as being the likely result of an error in spectral data collection.

The statistics obtained with the discriminant models assayed in terms of the coefficient of determination in cross-validation (1 − VR) and standard error of cross-validation (SECV) are shown in Table 1. There were clear differences in the data obtained depending on whether or not spectra were subjected to mathematical pretreatment (Derivative + SNVD). No significant differences were observed between the two models using derivative pretreatment, although statistics were slight better with first derivative pretreatment, with a coefficient of determination of *0.95* and a standard error of cross-validation of *0.133*.

A sample will be classified as uncertain within a class when the difference between the value given to that sample in its class and the value given in the nearest class is lower than the minimum difference (Pérez Marín, 2005).

Fig. 3 shows a 3D plot of the two populations as a function of scores for three principal components, in this case principal components 3, 4 and 5. It were selected scores 3, 4 and 5 to show the differences between both populations (MBM and PBF). The fraction of explainable variance in spectra for these scores was around 42–50% and 3D plot shows a clear spatial separation in the hypersphere of the two groups. Members of the MBM population lying to the left and those of the PBF population to the right. All the treatments assayed repeatedly yielded aberrant statistics for two particles belonging to the MBM set.

The first approach to choosing the best discriminant model was based on cross-validation statistics; however, the ability of the model to detect MBM contamination could only be evaluated by external validation using the two validation sets described in Section 2. All the discriminant models correctly predicted all validation samples (sets 1 and 2).

Table 2 offers a comparison of reference results and results obtained using each of the three best discriminant models developed and applied to samples from validation set 1. The results show that the best models were those

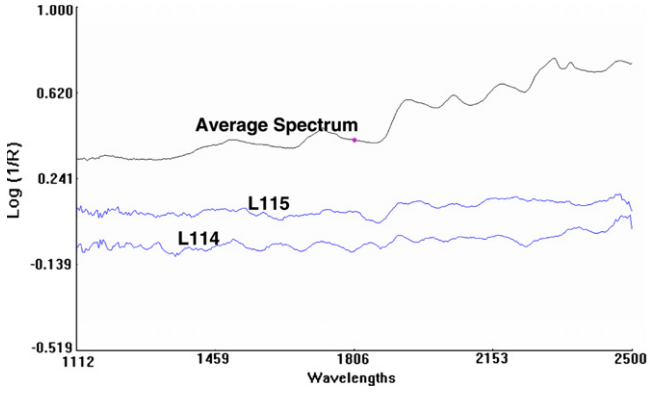

Fig. 2. Average spectrum of the calibration set from MBM population and spectra classified as outliers.

Table 1
PLS discriminant performance for meat and bone meal (MBM) adulteration of feedstuffs

Calibration		SEC	SECV	1 − VR
Scatter correction	Mathematical treatment			
None	0,0,1,1	0.165	0.168	0.88
SNVD	1,4,4,1	0.107	0.133	0.95
SNVD	2,5,5,1	0.112	0.119	0.94

SEC, standard error of calibration; SECV, standard error of cross-validation; 1 − VR, coefficient of determination in cross-validation; SNVD, standard normal variation and detrending.

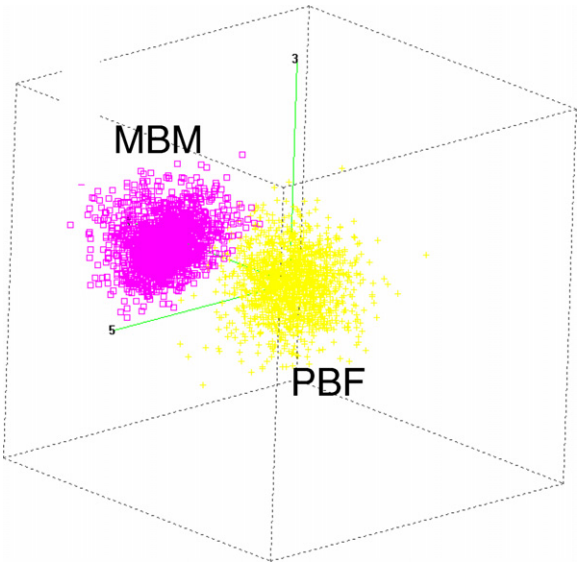

Fig. 3. 3D PCA scores plot of MBM and PBF spectral data (range 1100–2500 nm).

Table 2
PLS discriminant validation performance for feedstuffs adulterated with high percentages of meat and bone meal (MBM)

Sample	Reference value (%MBM)	Mathematical treatment					
		0,0,1,1		SNVD 1,4,4,1		SNVD 2,5,5,1	
		% PBF	% MBM	% PBF	% MBM	% PBF	% MBM
1	0[a]	82.20	17.80	82.49	17.51	81.92	18.08
2	0	99.62	0.38	100.00	0.00	100.00	0.00
3	0	100.00	0.00	100.00	0.00	100.00	0.00
4	0[a]	99.41	0.59	99.80	0.20	99.41	0.59
5	32.1	94.79	5.21	90.73	9.27	91.70	8.30
6	26.7	93.68	6.32	93.68	6.32	93.49	6.51
7	27.4	93.54	6.46	94.72	5.28	93.93	6.07
8	33.4	94.95	5.05	93.12	6.88	93.58	6.42
9	27.7	98.24	1.76	93.93	6.07	93.93	6.07
10	34.95	86.85	13.15	86.13	13.87	83.06	16.94
11	0[a]	99.09	0.91	99.82	0.18	99.64	0.36
12	29.4	96.35	3.65	95.58	4.42	96.35	3.65
13	27.8	95.24	4.76	92.95	7.05	93.14	6.86
14	0	100.00	0.00	100.00	0.00	100.00	0.00
15	0	100.00	0.00	100.00	0.00	100.00	0.00
16	0	99.80	0.20	100.00	0.00	100.00	0.00
17	0	100.00	0.00	100.00	0.00	100.00	0.00
18	0[a]	80.75	19.25	81.19	18.81	81.49	18.51

Set 1. MBM, meat and bone meal; PBF, plant based feed; SNVD, standard normal variate and detrending.
[a] With added animal fat or blood-positive for reference technique.

which involved pretreatment of the spectral signal using a derivative and SNVD. Moreover, no particles were predicted as uncertain by either of these models.

Results agreed perfectly with reference data; the possible false positives yielded by the discriminant models (samples 1, 4, 11 and 18) were found by the official technique (classical microscopy) to contain animal blood. Given that the feeds in question contained animal fat, the blood probably came from fat impurities. Garrido-Varo et al. (2005) showed similar results by NIRS using the same external validation set.

However, although these 18 samples were representative of compound feeds commercially available at present, they were distributed into two separate, clearly-differentiated groups: non-contaminated samples, and feeds containing over 15% MBM. The next step in the validation of the NIRM method was therefore to evaluate the ability of the discriminant models to detected possible MBM contamination over a range of less than 1%. For this purpose, the second validation samples was used, comprising six samples containing less than 1% animal meal.

Classification of samples from external validation set 2 is shown in Table 3. As in the previous set, MBM particles were detected in all contaminated samples. Although MBM was not an ingredient of sample 24, this sample contained animal blood, and was therefore classified as contaminated within the set of MBM-contaminated samples. Previous works had demonstrated the capability of NIRM to detect concentrations around 0.05% (Baeten et al., 2005). Results obtained in this study show that developed methodology can be used to detect the presence of MBM at concentrations as low as 0.02% (see Table 3).

Although the results of external validation have been described in detail for each discriminant model, selection of the best model required analysis of the statistics taking into account total percentage of correctly-classified samples, number of false positives and number of false negatives. When using raw data (0,0,1,1) the model classified

Table 3
PLS discriminant validation performance for feedstuffs adulterated with low percentages of meat and bone meal (MBM)

Sample	Reference value		Mathematical treatment					
			0,0,1,1		SNVD 1,4,4,1		SNVD 2,5,5,1	
	% MBM	% Blood meal	% PBF	% MBM	% PBF	% MBM	% PBF	% MBM
19	1	0.2	84.00	16.00	94.00	6.00	95.27	4.73
20	0.5	0.02	98.20	1.80	99.10	0.90	99.25	0.75
21	0.02	0	95.82	4.18	98.80	1.20	98.21	1.79
22	1	0.02	99.41	0.59	99.80	0.20	99.80	0.20
23	0.25	0	99.40	0.60	99.80	0.20	99.40	0.60
24	0	0.3	97.73	2.27	99.09	0.91	99.77	0.23

Set 2. MBM, meat and bone meal; PBF, plant based feed; SNVD, standard normal variate and detrending.

8.31% as false positives. The best results were obtained by applying a first or second derivative pretreatment plus SNVD; these models yielded 100% correct classification of the validation sets used, with no false negatives or false positives.

It is important to remark that the development of methods using optical microscopy and PCR require analysts with experience. To this respect, methods based on DNA identification as PCR methodology have a risk of contamination elevated and at <1.0% >0.5% MBM contamination level, it seems mandatory to perform different replicates from the same extract, to draw any conclusion about compliance (Dardenne et al., 2004).

4. Conclusions

Taken together, all results of this study highlight the enormous potential of this method for the detection of MBM contamination in compound feedingstuffs. It has been confirmed the excellent capacity of NIRM methodology to detect unequivocally MBM particles in compound feeds.

Results obtained applying the developed methodology have showed an improvement compared with the tedious and time consuming official method for detecting ingredients of animal origin in compound feeds, because the proposed method eliminates the sample pretreatment avoiding the use of highly-toxic organochlorine solvents and the subjectivity of the analyst (NIRM works only with spectral libraries and spectroscopic data) and minimize the analysis time.

Moreover, sensitivity and specificity of the proposed qualitative method are according with the European Union legislation for analyzing food and feed safety.

Further work is in progress to extend libraries and evaluate the transference of spectral libraries between instruments located at different laboratories.

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