Discrimination of fish bones from other animal bones in the sedimented fraction of compound feeds by Near Infrared Microscopy (NIRM)

De la Haba M.J.[1], Fernández Pierna J.A.[2], Fumière O.[2], Garrido-Varo A.[1], Guerrero J.E.[1], Pérez-Marín D.C.[1], Dardenne P.[2] and Baeten V.[2]

[1] Faculty of Agriculture and Forestry Engineering. ETSIAM. University of Córdoba. 14071 Córdoba. Spain.

[2] Walloon Agricultural Research Centre (CRA-W), Quality of Agricultural Products Department, Chaussée de Namur 24, 5030 Gembloux, Belgium. (baeten@cra.wallonie.be)

ABSTRACT

Since the Bovine Spongiform Encephalopathy (BSE) crisis, the use of animal proteins in animal feed has been prohibited. From October 2003, the European Union (EU) adopted Regulation (EC) nº 1774/2002 governing Animal By-Products (ABPs), which seeks to address the possible risks inherent in recycling potential infectivity due to the absence of barriers within species, and to exclude the cannibalism which may be induced by intra-species recycling. There is an urgent need to develop fast and reliable methods for low-level identification of ABP origins. In this study, Near Infrared Microscopy (NIRM) was used to identify different classes of ABPs. Samples of fish meals (n=10) and meals of land-animal origin (n=50) were ground, sedimented and analysed using an Auto Image Microscope connected to a Perkin-Elmer Fourier Transform Near-Infrared Spectrometer (FT-NIR). Sediment fraction particles were spread on a Spectralon plate, presented to the NIR microscope and scanned in the 1112-2500 nm region. The Support Vector Machine (SVM) algorithm was used to construct models to identify class origin. Models correctly classified 100% of the samples in the calibration set and between 95 and 95.5% in the validation set. The results demonstrated the potential of FT-NIRM as a rapid method for distinguishing between fish and land-animal particles.

Keywords: *animal by-products, near infrared microscopy (NIRM), discrimination, class origin, Support Vector Machines (SVM).*

INTRODUCTION

Rendering is the conventional process for obtaining animal by-products by heating and evaporating the water contained within the tissues. Heating facilitates fat separation and provides sterilizing effects[1]. This method has proved to be one of the most efficient for the processing of raw animal by-products. Rendering, which utilises a time/temperature control process, preserves the nutrient contents of the derived ingredients[2]. Rendered animal by-products are generally classified as either *animal proteins* or *fats*. Meat and bone meal (MBM) is one of the most important *animal protein* products and is defined by the EU as *'the product obtained by heating, drying and grinding whole or parts of warm-blooded land animals from which the fat may have been partially extracted or physically removed. The product must be substantially free of hooves, horn, bristle, hair and feathers, as well as digestive tract content'*.[3] More specific definitions can be found in the Annex of the Processed Animal Protein (PAP) legislation,[4] in the Regulation governing Animal Protein By-Products (ABPs)[5] or in the AAFCO definitions.[6]

Prompted by the Bovine Spongiform Encephalopathy (BSE) crisis, the EU introduced in 1994[7] a ban on ruminant feedstuffs containing protein derived from ruminant tissues. This ban stated that European member states might be authorized to allow the feeding of proteins from other species, as long as a system was enforced which made it possible to distinguish between *animal proteins* of ruminant origin and those of non-ruminant origin. The feeding to ruminants of animal proteins from species other than ruminants was permitted.[7] Due to renewed outbreaks of BSE in the late 1990s, a Council Decision was finally taken in 2000,[4] which prohibited the use of all processed animal proteins for the feeding of farm animals kept, fattened or bred for the production of food. This Council

Decision also bans the feeding of ruminants with fish meal. The EU defines fish meal as *'the product obtained by processing whole or parts of fish from which part of the oil may have been removed and to which fish soluble may have been re-added'*.[3] The use of pure fish material in the animal production chain poses no risk and it is accepted that fish do not carry TSE. The European Commission legislation in force requires the use of methods to check for the presence of ruminant tissue in fish meal or in compound feeds containing fish meal. This is mandatory in order to allow fish meal to be used in ruminant feed.[8] In addition, the Animal Protein By-Products Directive also stipulates that species-specific bans (avoiding cannibalism) will be introduced once methods allowing the detection and identification at species level of animal by-products included in compound feed are available.

Current methods for the identification of animal species in feed and food are based on the analysis of protein (using mainly inmunological and electrophoretic methods), DNA (including DNA hybridization and PCR amplification protocols) and animal particles (by optical microscopy).[9] Optical microscopy, the official method for feed identification in the EU,[10] can distinguish the bones of land animals from those of fish. However, the technique is strongly dependent on the experience of the analyst, and does not enable differentiation of the various mammalian and bird species.[11] A number of studies have sought to ascertain the ability of optical microscopy to distinguish between material of land-animal and fish origin.[11,12] These studies, conducted on behalf of the DG Health and Consumer Protection (SANCO) or within the framework of the STRATFEED project,[13] have shown that detection of mammalian and avian meal at low levels (i.e. 0.1 %) in the presence of 5% of fish meal could pose problems for the official laboratories. They also show that optical

microscopy is not the perfect tool for distinguishing between mammalian and avian origins in animal feed ingredients.

Several studies testify to the ability of NIR spectroscopy to identify and/or quantify animal ingredients in a feed mixture.[14-27] Recent developments have lead to the use of combined instruments, e.g. a near infrared spectrometer attached to a microscope. In Near Infrared Microscopy (NIRM) the infrared beam is focused using a dedicated microscope on each particle of a sample spread on a sample holder, and the near-infrared spectrum is collected. The identification of the ingredients is made using the spectral features measured in the near-infrared region (1100-2500 nm) of the electromagnetic spectrum.[28,29] Piraux and Dardenne[30] demonstrated the potential of NIRM for the detection and quantification of MBM in feedingstuffs. They constructed spectral libraries including thousands of spectra of single particles from animal and vegetal ingredients and used them to discriminate between particles from authorised and unauthorised feed materials. They also concluded that around 600 particles should be analysed in order to detect at least one MBM particle with a probability of 95% in a 0.5% adulterated feed. Other studies suggest that 3000 particles need to be analysed in order to detect at least one MBM particle with a probability of 95% in a 0.1% adulterated feed.[31-33] It takes over three days to analyse this huge number of particles.[33] In order to increase the speed of analysis, and to decrease the limit of detection, the addition of a sedimentation step has been recommended by Baeten et al.[33,34]. Within the framework of the STRATFEED project[13] a complete NIRM protocol has been developed and validated to analyse the raw and sediment fractions of feedingstuffs. Application of this NIRM method to the sediment fraction enables detection of low levels of MBM in compound feed. Comparison with the optical microscopy method has shown no differences between the results obtained using the two techniques.[34]

The aim of this study was to ascertain the ability of the NIRM method to discriminate, in the sediment fraction, between bone particles of fish origin from those of land-animal origin (mammalian meat and bone meal, or MMBM). This is mandatory in order to ensure the proper application of the NIRM method within the framework of EC legislation.

MATERIALS AND METHODS.

Samples

The sample sets used to calibrate the NIR microscope comprised feed ingredients and feedstuffs selected from the CRA-W sample bank and from the STRATFEED sample bank stored at the University of Córdoba (Spain). The sets included samples of animal, vegetable and mineral origin. Most samples were produced and collected during the 2000–2002 period and analysed by other techniques, such as optical microscopy and PCR, which permitted confirmation or correction of the declared composition. Pure cattle and pig meat and bone meals were obtained from a pilot plant and produced from pure by-products of each animal species studied (cattle, sheep, pig, chicken). The material was treated at 133°C and 3 bars for 20 minutes, then dried under atmospheric conditions until the moisture content was below 10%. Finally, the product was pressed and ground. Samples were analysed by PCR in order to identify the species included in the sample. The results indicated compliance with the labelling of the materials used in this study. Pure fishmeal materials were obtained directly from fishmeal producers at various geographical locations (Norway, Chile, Denmark, Iceland, Peru and the United Kingdom), and purity was checked by PCR. The results indicated that fish meals contained no bovine, porcine or poultry materials. A total of 10 pure fishmeal samples and 50 MMBM samples were available.

Sample preparation

Prior to analysis, samples were sedimented as proposed by Baeten et al.[34] For this purpose, samples were ground with a RETSCH ZM100 grinding mill (Retsch GmbH, Germany). A total of 3 g and 10 g were sedimented for the animal ingredient and for the compound feed, respectively. The ground sample was placed in a separation funnel in 50 ml tetracholorethylene (TCE, $Cl_2C = CCl_2$). The funnel was shaken firmly for 30 seconds and then the walls were rinsed with TCE in order to wash down all the material. Two different phases were obtained: the “floating part”, containing the main part of the sample and the "sediment part”, containing particles of a density higher than 1.62, i.e. mainly bone particles, egg shells and minerals. The latter fraction was recovered on cellulose filters and dried for 2 hours. It was then weighed and stored in a clearly-identified glass bottle.

Instrumentation and spectrum collection

An Auto Image Microscope connected to a Perkin-Elmer Fourier Transform Near-Infrared Spectrometer (FT-NIR) was used. This instrument enables the collection of spectra from small surfaces (50µ x 50µ). The microscope includes a camera and a viewing system to magnify the visible light image of the sample, as well as to highlight and isolate a point of interest. The particles of the sediment fraction were spread on a spectralon plate and presented to the NIR microscope. Using the microscope pointer, the infrared beam was focused on each particle and the NIR spectrum (1112-2500 nm) was collected. This study used a resolution of 4 cm^{-1}, a gain of 4 and a total of 10 co-added scans. Spectra were obtained from the ratio between raw spectra and the background, consisting in the measurement of the spectralon plate. Perkin-Elmer autoimage software v.3.2.1 was used for spectrum collection and storage.

Perkin-Elmer “Convert Abs NM” software was used to convert the frequencies of the spectra from wavenumber (cm^{-1}) to wavelength (nm), and the Perkin-Elmer Specconv v.1.0

software was applied to convert spectra to an ASCII format. ASCII data were imported using MATLAB (The Mathworks Inc., USA).

SVM

The support vector machines technique (SVM) is widely used as a learning algorithm due to its high generalisation performance, offering a flexible and easy-to-compute solution.[35,36] Use of the SVM learning algorithm for classification purposes has received considerable attention in the chemometrics-related literature over recent years. It has been described in depth by Vapnik[37] and has been put forward as a method of solving the problems posed by the complexity of NIR spectroscopic data.[35,36]

SVM analysis is a learning algorithm that can perform binary classification by nonlinearly mapping n-dimensional input space onto a high-dimensional feature space. In this high-dimensional feature space, a linear classifier, or non-linear kernel classifier, is constructed, and the model is used to discriminate samples belonging to two different groups. Thus, an SVM learns to discriminate between the members and the non-members of a class (in the case of binary classification, the space contains only two elements, one of which is understood as the positive class (or +1) and the other as the negative class (or –1)). After learning the features of the class, the SVM recognizes unknown samples as a member of a specific class.

For a group of training samples (x_i,y_i) with $x_i \in R^n$ which belong to a class labelled by $y_i \in \{-1,+1\}$, the main equation for SVM is as follows:

$$\begin{cases} min\left[\frac{1}{2}\|w\|^2 + C\sum_{i=1}^{n}\xi_i\right] \\ \forall i,\ y_i \cdot (w' \cdot x_i + b) \geq 1 - \xi_i \end{cases}$$

where $\xi_i = |1 - y_i f(x_i)|$ is a non-negative slack variable which estimates classification error (relative to the decision function $f(x_i)$) at point (x_i,y_i). Errors are found when $\xi_i>1$. The first part of the equation tries to maximize the margin between the two classes, while the second part forces the optimisation towards minimal error through the user-selected regularisation parameter C. C is a constant that controls the trade-off between complexity and misclassification. The classification is linearly done by mapping the training data onto a higher-dimension feature space, Φ. In practice, it does not involve any computations in that high dimensional space. By the use of kernels, all necessary computations are performed directly in input space. A kernel specifies an inner product in feature space:

$$k(x_i, x_j) = (\Phi(x_i) \cdot \Phi(x_j))$$

An example of such a kernel is the Radial Basis Function (RBF):

$$K(x, x_i) = \exp\left(-\frac{\|x - x_i\|^2}{2\sigma^2} \right)$$

where σ represents the width of the Gaussian function and reflects the degree of generalisation.

Finally the decision function $f(x)$ is found, in the form:

$$f(x) = sign\left(\sum_{i=1}^{m} y_i \alpha_i k(x, x_i) + b \right)$$

The points x_i with $\alpha_i >0$ are called support vectors.

Model validation

Constructed SVM models have to be validated in order to estimate performance measures, in the case of classification learning, these refer to the misclassification rate. Here, two different procedures were selected for model validation.

For the first validation procedure, the set of available input-output measurements was divided into two parts - one part for training and one for testing, thus enabling comparison of the performance of different models constructed using the the training set, on the test set. The second validation procedure used was the well known leave-one-out cross-validation, which is the most widely used internal estimator. In classical regression, the leave-one-out cross-validation consists in splitting the learning data set, comprising L patterns, into a training set of size L-1 and a test of size 1, and averaging the squared error on the left-out pattern over the L possible ways of obtaining such a partition. In the case of classification, a misclassification ratio can be calculated for each sample left out.

The main disadvantage of this leave-one-out validation procedure is that all the data has to be used for training, and thus the procedure does not provide a true independent assessment of the quality of the model. Also, when using leave-one-out cross-validation, no one global model is obtained but L models are obtained. For these reasons, both validation procedures were used in this study and a final model was constructed using all the data and reporting statistics for both procedures.

RESULTS AND DISCUSSION

Spectrum selection

For the samples included in this study, a selection procedure comprising several steps was performed. When the samples were measured using NIR microscopy, the number of spectra obtained for each of them was different. The total number of spectra for all samples was 7259. The first step in selection was to reduce the database to an homogeneous data set. This was done by performing a PCA on the original data in order to select the first 10 PCs accounting for the maximum variance of the data. The Mahalanobis distances between the

objects in this new space (the PC space) were calculated, and only 75% of the samples with the lowest distance were selected. This procedure guaranteed a data set with no outliers. Taking these spectra, a second selection step was performed using the duplex design proposed by Snee[38] so that only 30 spectra were selected for each sample. The final selected data set thus comprised 2010 spectra, of which 510 were fish meal spectra.

To validate the results obtained, and thus the rate of discrimination between fish meal and other spectra, a validation data set was required. For that purpose, the data set was split into a calibration set (used to construct the model) and a validation set. This was done in a random manner, ensuring that at least 30% of each group (i.e. fish and non-fish) was present in the validation set. The final dataset consisted of a calibration set with 1380 spectra, of which 420 were fish spectra, and a validation set comprising 630 spectra, of which 90 were fish spectra. The calibration set was used to construct the fish vs. non-fish equation.

Spectral features

Figure 1 shows the mean NIR spectra of fishmeal and land-animal meal samples used. Both spectra presented similar absorption bands when displayed in the Log (1/R) form. Absorption bands at 1490 nm related to O-H stretch first overtone and to N-H stretch first overtone. However, this peak was stronger in land-animal meal spectra. At 1944 nm there was an absorption band related to water absorption bands (O-H stretch second overtone) and at 1726 another one related to C-H stretch first overtone associated with oil content and types. Bands at 2058 and 2174 nm were related to the peptide absorption of the amide group and had a high correlation with either crude protein or total volatile nitrogen content in fishmeal. Bands at 2306 and 2348 nm were related to C-H bond second overtone bands corresponding with protein and oil contents.[39] The results relating to fishmeal absorption bands agreed with those obtained by other authors using near infrared spectroscopy.[40-41]

Figure 1

Figure 2 shows the same spectra but represented as the second derivative of Log (1/R). Second derivative spectra displayed a trough corresponding to each band in the original spectra[39]. At 1714 nm the bands were related with C-H first overtone and at 2058 and 2168 nm with peptide absorption bands. Here, discrimination between spectra was possible at around 1720 nm, corresponding to the oil absorption band[42].

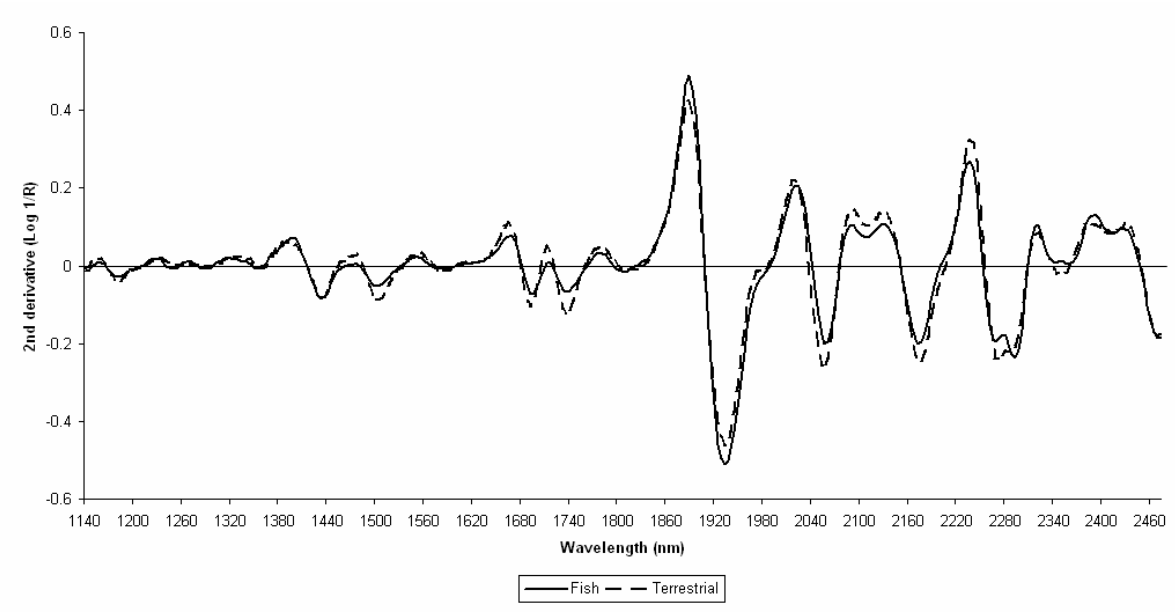

Figure 2

The small shifts in wavelengths and in absorbance values observed between the different samples highlight the discriminatory potential of the NIRS method.

Model construction and validation

The SVM fish *vs.* non-fish model was constructed using the calibration data set with C= 1000000 and σ=4.5 calculated using a grid search as proposed by Chih-Wei et al.[43]

The model yielded a classification rate of 100% (based on the calibration set) and its prediction ability reached an average correct classification of 95.5 % for the test set. Table 1 shows the success rate in confusion matrices obtained for both data sets. The success rate is defined as:

$$success\ rate = \frac{\left(\sum_{i=1}^{K} \frac{correctly\ classified\ samples\ in\ class\ \ i}{total\ number\ of\ \ samples\ in\ class\ \ \ i} \right)}{K} \text{ X } 100$$

with *K* being the number of classes. A success rate of, for instance, 92 indicates that 92% of the objects are correctly classified.

Calibration Set			**Validation Set**		
Belonged to...	**Classified as...**		**Belonged to...**	**Classified as...**	
	Fish	**MMBM**		**Fish**	**MMBM**
Fish	100	0	**Fish**	92	8
MMBM	0	100	**MMBM**	1	99
Success rate = 100%			Success rate = 95.5%		

Table 1

Table 2 shows the results when applying the leave-one-out validation procedure on the calibration set and also the results for the validation set.

Calibration Set			**Validation Set**		
Belonged to...	**Classified as...**		**Belonged to...**	**Classified as...**	
	Fish	**MMBM**		**Fish**	**MMBM**
Fish	100	0	**Fish**	91	9

MMBM	0	100	**MMBM**	3	97
Success rate = 100%			Success rate = 95%		

Table 2

In both cases the results show that a clear discrimination is possible between fish meal and other species, given the high success rate obtained in both cases.

Two independent sets were also predicted. The first set comprised 3 sediment samples from a ring trial at the Danish Plant Directorate, PDIR, Denmark and from the Veterinary Laboratories Agency, VLA, UK. The first sample (A) was "pure" fish meal containing 0.1% MMBM, the second (B) was a mixture of 0.1% fish and 2% MMBM and the third (C) was 0.1% MBM with no fish meal. The samples were first analysed at CRA-W by classical microscopy; in all cases samples were correctly detected. Then NIRM and the SVM models were applied. Table 3 shows the results as number of particles detected as fish or as MMBM for all samples after application of the two equations (fish vs. non-fish and MMBM vs. non-MBMM). It can be concluded that NIRM was able to detect the presence of fish in samples A, B and G.

Sample	Composition	Analysed particles	Detected fish particles	Detected MMBM particles
A	Pure fish + 0.1% MMBM	344	145	152
B	0.1% Fish + 2% MMBM	332	3	5
C	0.1 % MMBM	315	0	6
D	0.1 % MMBM	157	0	11
E	0%	360	0	0
F	0.5 % MMBM	216	0	31
G	0.1% MMBM + 1 % Fish	493	23	30
H	0.3% MMBM	400	0	45

Table 3

Conclusion and perspectives

The results show that NIRM combined with SVM can be used for the detection of MMBM with a high success rate. In contrast with optical microscopy, this method offers one main advantage: it is not dependent on the subjectivity of the analyst because particles are identified from their NIR spectral fingerprint and not by visual inspection. Further developments will focus on extending libraries with more land-animal and fish particles and also on the selection of new markers for the discrimination of animal species for each particle.

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Table Captions

Table 1 - Success rate in confusion matrices obtained for calibration and validation sets

Table 2 - Success rate in confusion matrices obtained for calibration and validation sets when applying leave-one-out cross validation

Table 3 – Results for external validation sets (samples A-H)

Figures Captions

Figure 1- Near infrared reflectance spectroscopy (NIRS) mean spectrum of fishmeal and land-animal meal samples

Figure 2- Second derivative of NIRS mean spectrum of fishmeal and land-animal meal samples.

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