# Feed authentication by near infrared microscopy

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## Introduction

Bovine Spongiform Encephalopathy (BSE) is a fatal degenerative disease affecting the central nervous system of cattle. According to the generally accepted scientific explanation, the BSE epizootic in the United Kingdom has its roots in the recycling of contaminated cattle carcasses processed into animal feed in the form of meat and bone meal (MBM), as well as in changes made (in 1981–82) in the technological processes used in the production of such meal (reduction of drying temperatures and discontinuation of solvent defatting in order to optimise the extraction of fats).[1]

The Commission Decision 94/381/EC of 27 June 1994 has banned, with effect from 27th July 1994 in all the Member States, the use of proteins derived from ruminant tissue or—in the event of difficulty of identification—from any mammalian tissue for feeding ruminants. Moreover, the EU laid down compulsory manufacturing standards in all the Member States in order to improve the safety of meal for other animals (pigs, poultry, fish, etc.). These standards have been tightened since 1 April 1997 (Decision 94/449/EC of 18/7/96: minimum parameters for the processing of animal waste from mammals, excluding fats : Ø < 50 mm, t° > 133° C, t: 20, p: 3 bar).

The ban on the use of mammalian protein in the feeding of ruminants need fast and reliable analytical methods to identify animal ingredients in compound feed.

In most of the European countries, the microscopic method is currently adopted. The detection limit of the microscopic method is approximately 0.1% or even smaller. When used for quantification of animal ingredients in feedstuffs this method is dependant on the presence of bones in the product.[2] Moreover, the accuracy is very dependant on the bone content in the animal ingredient to be identified in a compound feed. Furthermore, the differentiation of bones from mammalians and poultry is very difficult and considerable expertise is necessary to make this differentiation.

Contrary to the feedstuff microscopy, the commercial Elisa can identify the different animal species depending on the available antibodies. The detection limit of commercial Elisa used for detection of constituents of animal origin in compound feedstuffs was at a level of approximately 5% depending on the animal species. When increasing the temperature treatment of the animal product the sensitivity of the detection decreases respectively. Identification of products heated to above 130°C could not be achieved.[3,4]

The DNA methodology is another approach for the identification of animal ingredients in compound feedstuffs. By using PCR procedures and appropriate primer pairs, the methodology allows a rapid and sensitive detection of species-specific DNA sequences from meat and bone meal. It allows detection of the presence of bovine derived meat and bone meal in feedstuffs containing less than 0.125% meat and bone meal.[5]

Using near infrared (NIR) is another possible way of identifying animal ingredients. The traditional application of NIR in the analysis of feeds has been focused on the development of predictive

Near Infrared Spectroscopy: Proceedings of the 9th International Conference

calibration equations relating spectral data to chemical or nutritional parameters (for example, crude protein, crude fat, fibre fractions, starch, digestibility, energy, etc.). In the particular case of ingredients recognition in a mixture, NIR has been used for a number of applications and seems able to predict accurately the ingredient composition of binary mixtures.[6] Further research is needed for the quantitative prediction of meat and bone meal in compound feedstuffs.[7]

In fact, at present, none of the methods described above is totally satisfactory to detect and to quantify meat and bone meal in compound feed. We present, hereafter, a new method, based on FT-NIR microscopy, to detect and to quantify meat and bone meal in compound feed. This spectromicroscopic method consists of the analysis of several hundreds of particles being the result of the grinding of a compound feedstuff. These particles are then identify as contaminant (meat and bone meal) particles or not by comparing their spectra with reference libraries. Finally, the area proportion of meat and bone particles found is related to the meat and bone meal percentage in the compound feedstuff.

## Materials and methods

### Perkin-Elmer NIR microscope

The AutoIMAGE Microscope is connected to a Perkin-Elmer FT-NIR and allows the collection of spectra from extremely small samples (up to 5 μ × 5 μ). The microscope includes a camera and a viewing system that magnifies the visible–light image of the sample to observe, to position (by means of a motorised sample stage with a minimum step size of 1 μ) and to isolate a point of interest. The image of the sample is displayed on a PC monitor. The AutoIMAGE software enables the control of the operation of the microscope, to map and to collect spectra from a sample. Spectra can be collected in reflectance or transmittance mode.

### Feedstuffs

#### *Raw materials*

Raw materials samples used to construct reference libraries were supplied, principally, by the Belgian Ministry of Small Enterprises, Traders and Agriculture as well as by two Belgian feed producers.

The complete set of forbidden raw materials for feeding ruminants consisted of:

- meat and bone meal (MBM) (15 samples)
- meat meal (MM) (13 samples)
- ground bones (4 samples)
- feather meal* (3 samples)
- poultry by-products* (8 samples)

* feather meal and poultry by-products are not forbidden for feeding ruminants but we can not actually differentiate these products from forbidden raw materials by NIR microscopy.

The complete set of allowed raw materials for feeding ruminants consisted of :

- fishmeal (19 samples)
- peas (6 samples)
- manioc (6 samples)
- wheat (2 samples)
- blood meal (1 sample)
- rape extracted oil cake (3 samples)
- corn (3 samples)
- maize gluten feed (1 sample)
- maize germ oilcake (1 sample)
- soybean (5 samples)

- flax (3 samples)
- lucerne (alfalfa) (4 samples)
- milk by-product (2 samples)

***Compound feedstuffs***

Compound feedstuffs with known concentration in MBM were used to build and to validate the model.

The training set consists of a basic, non-adulterated compound feedstuff composed by a Belgian feedstuff producer (1A, see Table 1 for composition) and different MBM thoroughly mixed in different weight proportions (0 to 10% in 2% intervals).

The test set consists in three compound feedstuffs. The first one was prepared by the State Analysis Laboratory, Tervuren and the other two were prepared by a Belgian feedstuff manufacturer.

**Table 1. Composition of the basic, non-adulterated, compound feedstuff 1A.**

| Feedstuffs |
|---|
| Palmist |
| Wheat |
| Flax |
| Soy bean |
| Citrus |
| Coconut |
| Glutenfeed |
| Sugar beet roots |
| Bran |
| Minerals |

## Sample preparation

Samples were ground with a 1mm hole sieve (Retsch mill, Germany).

## Sample scanning and spectra acquisition

Analyses were made on particles displayed on a reference surface (spectralon) in reflectance mode with an aperture size of 50 μ by 50 μ. Reflectance data as Log 1/*R* were recorded at 4 nm intervals over the region 1112 to 2500 nm, giving 348 data points per spectrum. Spectra were averaged from 100 scans for the libraries construction and from 10 scans for the compound feedstuffs analysis.

## Data treatment

Spectral data were processed using ISI software (NIRS 3 ver. 4.0 and WinISI, Infrasoft International, Port Matilda, PA, USA) and SAS software, ver. 6.12 (SAS Institute Inc., Cary, NC, USA). Images of particles were processed using Micro Image 3.0 (Olympus Optical Co., Hamburg, Germany).

Canonical discriminant analysis was used to derive canonical variates that summarise between-group variation in much the same way that principal components summarise total variation. The first canonical axis was used to visualise differences between groups.

Because each group (allowed particles and forbidden particles) is a mixture of different populations (different raw materials) it was difficult to assume multi-normal distributions. This was confirmed by the results of a normality test done on raw spectra for each variable: for the first group (allowed particles), the normality hypothesis were rejected at a level of 0.05 for all variables, and for the second group (forbidden particles), the normality hypotheses were rejected at level 0.05 for 291 variables. Therefore, we decided to use a non-parametric method to discriminate between groups. An artificial neural network (multilayer perceptron network with back proprogation based on the partial least squares scores) was used to discriminate between groups encoded as –1 for allowed particles and 1 for forbidden particles. Predicted values below 0 were assigned to the first group and values above 0 were assigned to the second group. Previously, data were processed using standard normal variate and detrend SNVD along with a first derivative math treatment 1,4,4,1.

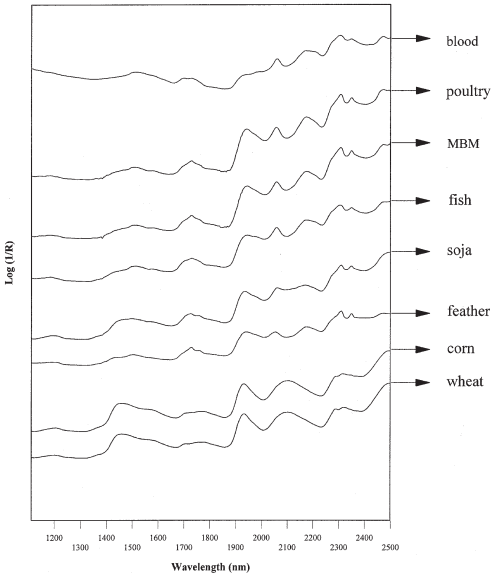


**Figure 1. Mean spectra of particles of characteristic raw materials.**

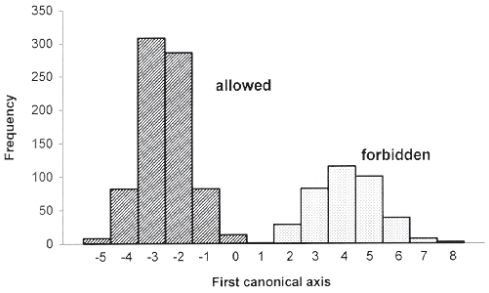


**Figure 2. Separation between groups along first canonical axis for calibration set.**

## Results

### Spectral features

The spectral features of the mean spectra of particles of the most characteristic raw materials are shown in Figure 1. Characteristic bands of water are observable at 1452 nm (OH first overtone) for plant raw materials and 1940 nm for all spectra. Soya and animal raw materials show bands at 2312 and 2356 nm (CH combinations) due to fat and bands at 2064 and 2184 nm (NH combinations) due to protein, whereas corn and wheat show a band at 2100 nm (OH combinations) due to starch.

There are great similarities between spectra of MBM, poultry, feather and fish which are not easily visually differentiable and which highlight the need to use chemometrics to distinguish between them.

### Qualitative analysis

A canonical discriminant analysis conducted on the calibration set allows the separation between the two groups [allowed and forbidden raw materials (Figure 2)].

A predictive discriminant analysis, using an artificial neural network (ANN), is used to classify particles into two groups on the basis of their absorbances from 1112 nm to 2500 nm, the first group is made of forbidden particles and the second group gathers allowed particles. A prediction rule is estab-

**Table 2. Repartition of the particles analysed during the construction and the validation of the discrimination rule.**

| | Number of particles (number of samples) | | |
|---|---|---|---|
| | Forbidden particles | Allowed particles including fish meal | Total |
| Construction of the discrimination rule | 379 (13) | 780–210 (26–7) | 1159 |
| Validation of the discrimination rule | 912 (31) | 960–360 (32–12) | 1872 |
| Total | 1291 (44) | 1740 (58) | 3031 |

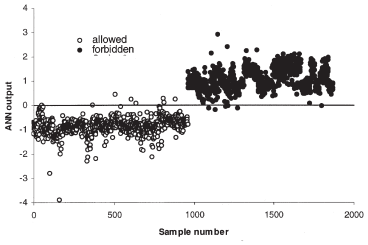


**Figure 3. Classification of allowed and forbidden raw material particles using an artificial neural network for the validation set. The horizontal line marks the threshold used to separate groups encoded as –1 for allowed particles and 1 for forbidden particles: ❍ allowed, Δ forbidden.**

lished with particles for which we know the group of origin (calibration or construction set). More than 3000 particles were analysed to construct and to validate the discrimination rule.

Table 2 shows the repartition of the particles analysed during the construction and the validation of the discrimination rule and Table 3 shows the results of the validation of this rule. Results of the ANN are shown in Figure 3 where the output of the ANN for each sample in the test set has been plotted against its arbitrary sample number.

The overall error rate, estimated with the independent validation set of particles, is given by :

Overall error rate = (0.63 + 0.66)/2 = 0.64%.

**Table 3. Validation of the discrimination rule.**

| | Number of particles classified into each group (percentage) | | |
|---|---|---|---|
| | "allowed" | "forbidden" | Total |
| Particles from the group "allowed" | 954 (99.37%) | 6 (**0.63%**) | 960 |
| Particles from the group "forbidden" | 6 (**0.66%**) | 906 (99.34%) | 912 |

**Table 4. Quantitative analysis. Results of the calibration step.**

| Sample number | % MBM (wlw) in sample | Particles analysed | | Particles identified as MBM | | | |
|---|---|---|---|---|---|---|---|
| | | nb | area | nb | % nb | area | % area |
| 0a | 0 | 601 | — | 0 | 0 | 0 | 0 |
| 0b | 0 | 600 | — | 0 | 0 | 0 | 0 |
| 2c | 2 | 667 | 549327 | 12 | 1.80 | 9383 | 1.71 |
| 2d | 2 | 627 | 599213 | 8 | 1.28 | 8102 | 1.35 |
| 4c | 4 | 599 | 626755 | 13 | 2.17 | 11980 | 1.91 |
| 4d | 4 | 604 | 649961 | 18 | 2.98 | 14011 | 2.16 |
| 6c | 6 | 620 | 585088 | 19 | 3.06 | 22535 | 3.85 |
| 6d | 6 | 634 | 549133 | 26 | 4.10 | 23081 | 4.20 |
| 8c | 8 | 617 | 631854 | 36 | 5.83 | 36003 | 5.70 |
| 8d | 8 | 618 | 542522 | 20 | 3.24 | 14764 | 2.72 |
| l0c | 10 | 623 | 530410 | 45 | 7.22 | 36396 | 6.86 |

### Quantitative analysis

#### *Calibration step*

To estimate the proportion of meat and bone meal in feed, compound feedstuffs with known concentrations of meat and bone meals were used to construct the calibration equation. The training set consisted of a basic, non-adulterated compound feedstuff and different meat and bone meals thoroughly mixed in different weight proportions (0 to 10% in 2% intervals). Image analysis was used to measure the area proportion of the meat and bone particles in the compound feed. Results are given in Table 4 and Figure 4 shows the relationship between the proportion of meat and bone meal in feed and the area proportion of the meat and bone particles.

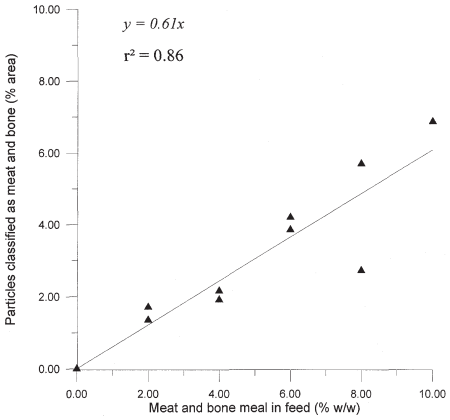


**Figure 4. Proportion of meat and bone meal in feed estimated by the area proportion of meat and bone particles.**

**Table 5. Quantitative analysis. Results of the validation step.**

| Sample number | Sample description | % Mbm (weigth) | % mbm estimated by NIR microscopy |
|---|---|---|---|
| 2a | pasture supplement | 2 | 2.02 |
| 3a | sow feed | 6 | 4.57 |
| 4a | pheasant feed | 6 | 3.36 |

#### *Validation step*

The validation step consisted of the analysis of four independent compound feedstuffs which range from 2 to 6% MBM. Results are given in Table 5.

## Conclusions

### Qualitative analysis

Results of the discriminant analysis between particles of raw materials allowed or forbidden for feeding ruminants indicate that it seems possible to detect, with NIR microscopy, MBM particles in a compound feedstuff with a success rate greater than 99%. These good results must be tempered for two reasons.

First, at present, it is difficult to differentiate between bovine meat meal particles and feather or poultry meat meal particles.

Secondly, if the MBM proportion in a compound feedstuff is low, we need to analyse a large set of particles if we want to observe at least one MBM particle with a high probability. For example, if there is 2% MBM in a compound feedstuff and if we want to observe at least one MBM particle with a probability of 95%, about 250 particles should be analysed. If there is 0.5% MBM in a compound feedstuff, which is the maximum acceptable level according to the opinion of the EC Scientific Steering

Committee[8] and if we want to observe at least one MBM particle with a probability of 95%, about 1000 particles should be analysed.

### Quantitative analysis

The results of the analysis of compound feedstuffs with known concentrations in MBM (Table 5), even if they are not sufficient to allow definitive conclusions, are promising. The accuracy obtained, is not sufficient to allow a quantitative control of compound feedstuffs. However, it certainly seems to be as good as the currently adopted microscopic method. Further analysis is required to improve this accuracy.

### Perspectives

The differentiation between bovine meat meal particles and feather or poultry meat meal particles could be achieved by using a larger spectral range (780–2500 nm or 400–2500 nm).[6,9]

The quantitative analysis is performed by a regression model related to the proportion of meat and bone meal in feed and the area proportion of the meat and bone particles. This model could be improved by discriminating between meat particles and bones particles. The discrimination between meat and bone particles could take into account density differences between these particles and therefore get a better quantitative model.

## Acknowledgement

This project was financed by the Ministry of Agriculture, DG4.

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