*Received: 8 August 2009* ■ *Revised: 1 February 2010* ■ *Accepted: 3 February 2010* ■ *Publication: 5 May 2010*

JNIRS JOURNAL OF NEAR INFRARED SPECTROSCOPY

# In-house validation of a near infrared hyperspectral imaging method for detecting processed animal proteins in compound feed

**Juan Antonio Fernández Pierna, Pierre Dardenne and Vincent Baeten***

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department, Henseval Building, Chaussée de Namur, 24, 5030 Gembloux, Belgium. E-mail: baeten@cra.wallonie.be

**This paper presents a framework for developing and validating a near infrared (NIR) hyperspectral imaging method as a standard protocol accepted by regulatory authorities. The focus is on detecting processed animal proteins (PAPs) in feedstuffs. Studies aimed at determining the performance characteristics and robustness of the protocol are presented. Various criteria and tests were used to assess the limit of detection, the repeatability and the risk of cross-contamination and to validate the NIR hyperspectral imaging method for detecting PAPs in compound feed. This protocol has been fully tested and validated though different studies in line with International Standard ISO 17025 and it is essential in order to transfer the method to other laboratories and introduce this technology to official control at the laboratory level.**

*Keywords*: NIR hyperspectral imaging, feedstuffs, PAP, BSE, ISO 17025

## Introduction

In the document *The TSE Roadmap*, the European Commission (EC) presents and discusses the next stages of the bovine spongiform encephalopathy (BSE) policy.[1] Since the earliest case of the BSE epidemic was confirmed in the UK in 1987, strict legislation has been enacted year after year to eradicate it in Europe.[2] This legislation has taken into account scientific and epidemiological evidence showing that meat and bone meal (MBM) is the most probable vector of the disease. The ban of ruminant feedstuffs containing protein derived from ruminant tissues was first introduced at European Union (EU) level in 1994.[3] Due to the resurgence of BSE problems at the end of the 1990s, a Council Decision was taken in 2000[4] which prohibited the use of all processed animal proteins (PAPs) for all farm animals kept, fattened or bred for food production. The EC is now considering amending certain measures which will not endanger health or the policy on eradicating BSE. The changes will consider whether the positive trend continues and if the right scientific conditions are in place. The trend of the BSE epidemic shows a clear improvement in the situation in recent years[5] because there has been a significant decrease in the number of cases of the disease in the EU (the annual incidence rate of BSE in the EU was of 31.09, on average, for 2002 versus 5.68 for 2008 according to the World Organisation for Animal Health). Also, the follow-up on positive BSE cases detected since 2001 indicates that the main contamination through feed occurred in 1994–1995, followed by a sharp decrease as a result of the BSE measures taken.

Changes to the rules on forbidden and authorised raw animal materials in feed formulations require the development of a strategy to assess the absence of illicit materials in the feed

ISSN: 0967-0335
doi: 10.1255/jnirs.872

mills and at the rendering plants. This strategy has to meet to the requirements of the control laboratories (for example, robustness, low limit of detection [LOD]) and of the people involved in the feed chain (i.e. it must be fast and cost-effective). Until now, the official controls have been based exclusively on the microscopic identification of illicit ingredients and, more particularly, on searching for bone and scale particles in the sediment fraction of the analysed feed. Microscope identification carries the possibility of identifying other specific particles (for example, muscle and hair) in the raw (i.e. non-sedimented) fraction (EC Directive 126/2003). Depending on the complexity of the feed material, the percentage of sediment and the need to discriminate fish material, the microscopic method takes about 90 min to detect the presence or absence of PAPs in the raw and sedimented fractions of a sample.

Several options have been studied in order to help the official and industrial control laboratories. These options can also be used to complement the strategy by allowing species-specific detection.[6] They include an increase in samples analysed by unit of time, the quantification of raw and sediment animal particles and the development of alternative methods based on molecular biology (for example, polymerase chain reaction [PCR][7–9]), immunoassay,[10,11] liquid chromatography,[12,13] and spectroscopy (for example, near infrared [NIR] spectroscopy[14–17]). The PCR method has the advantage of great species specificity when adapted primers for MBM detection are used. However, it has a limitation due to the possibility of cross-contamination and to the addition of permitted products (i.e. milk powder, blood and egg by-products). PCR, such as the immunoassay techniques, shows encouraging developments with regard to test heat-stability.[18] One of the best immunoassay candidates, because of its low cost and rapid screening method, is enzyme-linked immunosorbent assay (ELISA).[19] Some weakness, such as the loss of sensitivity with heat-treated animal meals and the cross-reactivity with some plant feed ingredients, limits the use of ELISA as a routine method for MBM detection. With the NIR method, promising results have been obtained, but the actual LOD is too high with regard to the safety requirements.[15,20]

Developments based on a combination of techniques have enabled powerful methods to be elaborated for the analysis of feed ingredients and compound feeds. In one case, a NIR spectrometer coupled with a microscope (NIRM) was successfully used to detect and quantify animal proteins in feed.[21–27] The NIRM installed at the CRA-W has operated under accreditation since 2005, following International Standard ISO 17025 (published by the International Organisation for Standardisation, Geneva, Switzerland). This method has also provided good results for detecting a series of feed ingredients of animal or vegetable origin, traditionally used in the formulation of feedstuffs.[22,28] The advantage of NIRM is that it combines the benefits of optical microscopy (detection based on the presence of animal particles not affected by the rendering process applied to the MBM) with the benefits of spectroscopy (detection based on the specific chemical composition of animal tissues and no need for a trained and skilled technician). The main limitation of NIRM is that it is slow because of the sequential collection of particle spectra. To overcome this problem, it has been suggested that work should be done on the sediment fraction, as with the reference method.[23,25] Although this approach boosts the method and partly reduces the problem of sampling with heterogeneous samples, the detection is based only on the presence of bones in the sediment fraction. This method gave conclusive results for detecting animal meal, but did not increase the speed of analysis compared with classical microscopy.

An alternative to NIRM is the use of a more recent technology called near infrared hyperspectral imaging. This technology has been described as a powerful approach for remote sensing in precision agriculture,[29] for forestry and environmental applications,[30,31] for mineralogy[32] and for military applications,[33] among others. The agro-food applications were reviewed recently.[25,34–37] The success of NIR imaging stems from a combination of factors: high-performance and uncooled NIR sensitive focal plane array detector, digitally tuneable infrared optical filters, the significant increase in computer speed and the capacity of laboratory computing platforms.[38] The integration of these elements has shown promising results in the determination of quality parameters for complex matrices such as pharmaceutical blends[39,40] and in the detection of apple surface defects and contamination.[41] It also allows spatial and spectral (and therefore chemical) information characterising the samples to be obtained at the same time. A recent study has shown that the combination of NIR imaging spectroscopy and some non-linear chemometric classification techniques could allow a regulatory laboratory to certify and quantify the presence of MBM in compound feed.[42] Another study has been conducted to prove that this technology allows complete screening of feedstuffs, while also providing an attractive solution for characterising feed mixtures.[43] One of the great advantages of this method is that it allows the detection of particles of bones and muscles (the only method able to do it). In microscopy, the work is done in particles after a sedimentation step. With NIR hyperspectral imaging, bones and muscles can be measured simultaneously without the need for a sedimentation step.

Validating NIR hyperspectral imaging for detecting PAP in compound feed is necessary in order to demonstrate the robustness of the protocol. Previous work has involved in-house validation for detecting PAPs in compound feedstuffs using NIR chemometric models[44] and in-house validation of NIRM.[45] This paper describes an in-house validation of the NIR hyperspectral imaging method for qualitative PAP detection, in line with the International Standard, ISO 17025. The work included the practices applied to an NIR imaging system for detecting PAPs in compound feed which seeks to determine the performance characteristics, to ensure the correct implementation of the method in control laboratories and to provide a framework in which the method can be developed and validated as a standard protocol accepted by feed control laboratories. The in-house validation presented in this

paper led to ISO 17025 accreditation for the NIR hyperspectral imaging system used in this work.

# Materials and methods

## Description of the near infrared imaging system

The NIR camera used in this study was a MatrixNIR™ Chemical Imaging System (Malvern Instruments, Analytical Imaging, Columbia, MD, USA). This instrument includes an NIR imaging system, a power supply and a workstation. It was operated using the Matrix Acquire from Malvern Instruments, and enables NIR images from 0.2 $cm^2$ to 4.4 $cm^2$ to be taken. For this study, only the 4.4 $cm^2$ configuration was used. Four tilting lamps (6.200 A current and 7.015 V voltage) illuminated the sample holder fixed on a microscopic rack, allowing it to move in the three dimensions. The power intensity of the lamps could be adjusted from 0 to 90 W. The reflected light was collected by optics and directed towards two coupled liquid crystal tuneable filters (LCTFs) that allowed the reflected energy to pass through sequentially within a defined wavelength range. Two LCTFs were used in order to sharpen the bandwidth. They were adjusted to collect the energy in the 900–1700 nm spectral range with a resolution of 10 nm (81 data points). After the LCTFs, the reflected energy then passed through infrared focal plane arrays, 240×320 in size, corresponding to 76,800 individual infrared detector elements (or pixels). For each pixel, the compilation of the absorbances at each wavelength gave a spectrum. The number of coadds and scans were 16 and 4, respectively. Both the spectra and the images were saved in a compressed format (spf) that was compatible with ISys software (Malvern Instruments) and easily transferable to Matlab 7.3 (The Mathworks Inc. Natick, MA, USA).

Depending on the size of the particles used in this study, between 250 and 350 particles were analysed at the same time in one image. All the information obtained was summarised in three kinds of spectral cubes: background, dark and sample spectral cubes. The background image was collected using the same conditions as used for the sample, but with the sample replaced with a piece of high reflectance white ceramic.[46] Dark scans are easily collected by closing the field of view, which is equivalent to having no sample. Then, the hyperspectral reflection cube of each image corresponded to the logarithm of the ratio between both cubes, as follows:

$$\text{reflection spectral cube} = \log\left(\frac{\text{sample spectral cube} - \text{dark spectral cube}}{\text{background spectral cube} - \text{dark spectral cube}}\right) \quad (1)$$

## Description of the feed materials

The selection of samples is critical for the success of a validation study. The samples used for the validation step of this study were carefully selected and came from several EU projects and inter-laboratory studies: Stratfeed (Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedstuffs);[47] Nutreco Holding NV, IAG (International Association for Feedingstuff Analysis); VLA (Veterinary Laboratories Agency), DG Sanco (Directorate General for Health and Consumer Affairs)[48–50] as well as from the CRL-AP sample bank hosted at CRA-W. All the samples had been collected and analysed by the Community reference laboratory to assess the composition. For this, classical microscopy and DNA analyses (PCR) were used. The samples were selected to be representative of the different products that can be found in compound feed. Some are mixtures of MBM prepared from different batches and sources of controlled materials

For this work, particles were collected from 26 pure animal meals and 59 pure vegetable meals and the mean spectrum was drawn from each particle driving to a training set of 5521 spectra, i.e. 2233 animal and 3288 vegetable particles. The database details have been described elsewhere.[42,43] It could be interesting to remark that 85 samples might seem to be rather limited when working with classical NIR spectroscopy, but when working with NIR imaging, it is also important (and maybe more important) to take into account the number of spectra extracted from individual particles and from different images because these cover the spectral variability included in the diversity of the particles (for example, feather, muscle, bones) making up each sample as well as the variability observed between analyses. This is the basis of the approach of NIR imaging for the detection of MBM.

Prior to NIR imaging analysis, the samples were sieved and only particles from the 250 µm to 1000 µm fraction were analysed. They were spread (to an approximate depth between 250 µm and 750 µm) on a binocular microscope (magnification factor 10) sample holder consisting of a ceramic plate of 2 mm thickness. For this in-house validation, the samples were measured using the NIR imaging system as they are (raw fraction) and also after an intermediary step of sedimentation as described in the official method (EC Directive 126/2003). The goal of the sedimentation was to concentrate the bone fragments in a reduced fraction in order to decrease the number of particles to analyse and to accelerate the analysis. This procedure is commonly applied with the optical microscopy method and involves the decantation of 10 g of compound feeds in tetra-chloroethylene in an appropriate funnel. This solvent has a density of 1.62 which allows concentration of the bones, fish bones, scales, egg shells and minerals while most of the plant particles float. The sediment was dried at room temperature before NIR imaging analysis.

## Methodology

After the samples had been measured with the NIR hyperspectral camera, the spectra obtained were checked in order to detect the presence of animal proteins. For this a procedure has been developed in order to obtain a fast and reliable method for detecting PAPs in feedstuffs using chemometric and visual tools. It was applied for both the raw and sediment fractions and only for particles larger than 250 µm. The

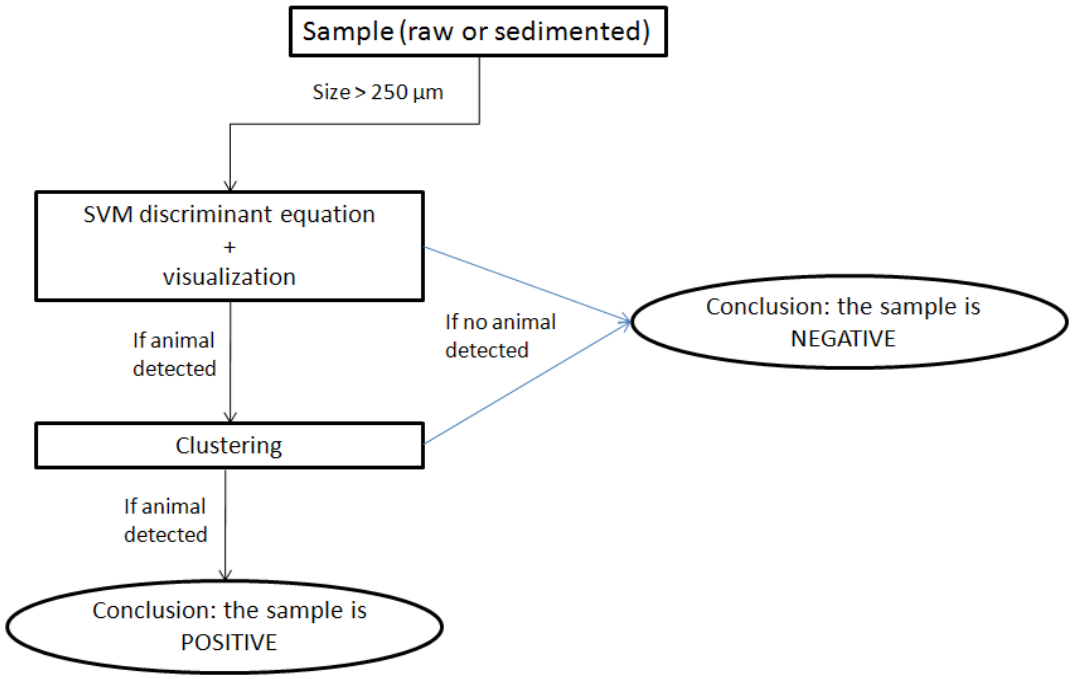


**Figure 1. Protocol flow chart showing the different steps performed to analyse samples in the fractions higher than 250 µm.**

procedure is represented as a flow chart in Figure 1. It consists of several steps.

The first step of the procedure consists of the application of the model equation to discriminate between animal and vegetable particles. To construct this equation, the support vector machines (SVM) method was selected because of its powerful characteristics as a discriminant technique (dealing with non-linearity, reproducibility, robustness); the method has been described in detail elsewhere.[42,43,51,52] The algorithm used was the Lin's Lib SVM v2.33 algorithm.[53] The discrimination model is based on a complete spectral database, which covered the variability of the main materials used in the formulation of compound feeds. The samples were selected from the sample bank at the CRA-W[22] and from the European sample bank constructed under the Stratfeed project "Development and validation of methods for the detection and quantification of mammalian tissues in feedingstuffs".[47]

Figure 2 shows the average spectra of a vegetable and animal (MBM) ingredient. These spectra have to be handled as fingerprints based on the chemical composition of the measured particles.

Figure 3 shows an example of the reconstructed image, with the results generated after applying the SVM discrimination model. The figure includes: (1) the pixels detected as containing processed animal proteins by the SVM model (indicated in black) and (2) the pixels with a maximum peak of absorbance (log [1/*R*]) at 1500 nm and absorbance higher than 0.1 (indicated in grey). The second kind of pixels has been selected because, by experience, some particles (mainly fish) present a peak at 1500 nm that was not always recognised by the model. The combination of both results should have detected the presence of most of the animal particles included in the image.

The procedure also included visual information that can be obtained by selecting the spectra of the suspicious pixels (via a computer mouse). This gave a visual confirmation of the results. Using this functionality of the procedure, a new figure appears showing different plots (Figure 4). The first plot shows the raw spectra {absorbance [log (1/*R*])} of the selected pixels at all the wavelengths. A spectrum of referenced pure animal (dashed line) was added as a target standard. The second plot shows the Mahalanobis distances for each of the selected spectra to the mean spectrum of the animal group in the calibration set. The third plot shows the Mahalanobis distances for each of the selected spectra to the mean spectrum of the vegetable group in the calibration set. In the case of the spectra selected here as examples, all the particles were animal meal according to the shape of the spectra and the distances.

However, if no pixels containing processed animal proteins were detected in the whole image after this first step, the sample can be concluded to be animal-free.

If pixels were detected with processed animal proteins, a second step was conducted in order to confirm the results obtained from the first step. It involved applying a clustering study on the results obtained with the discriminant SVM equation. The algorithm used was a density-based clustering approach called OPTICS[54] which was able to reveal and visualise clusters of arbitrary shapes and densities in a multivariate space. This technique allowed a deeper insight into

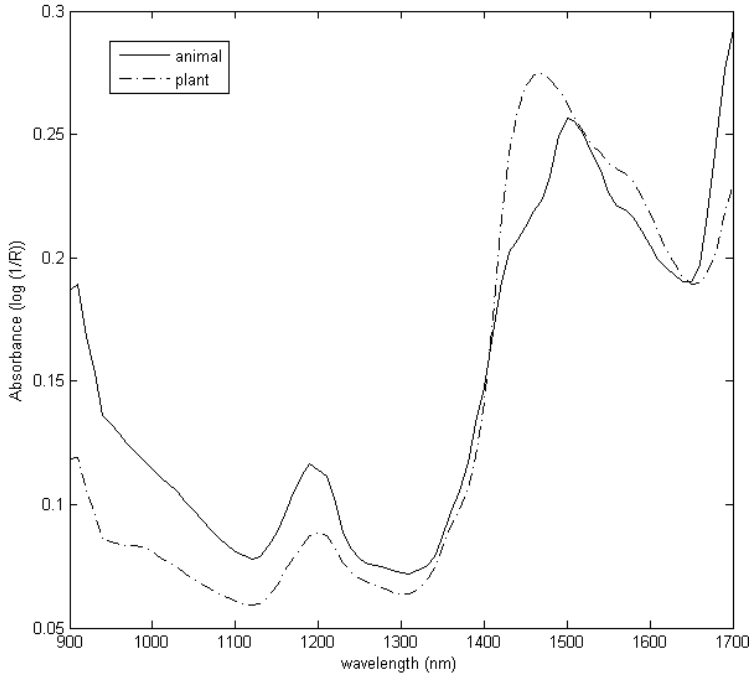


Figure 2. Mean NIR imaging spectra of plant and animal particles.

the obtained results by representing clusters of objects and removing individual pixels. If the suspected sample remained positive after this program and plot visualisation, this indicated the presence of PAP in the sample. If, after this step, no animal particles were detected, it was concluded that the sample was animal-free.

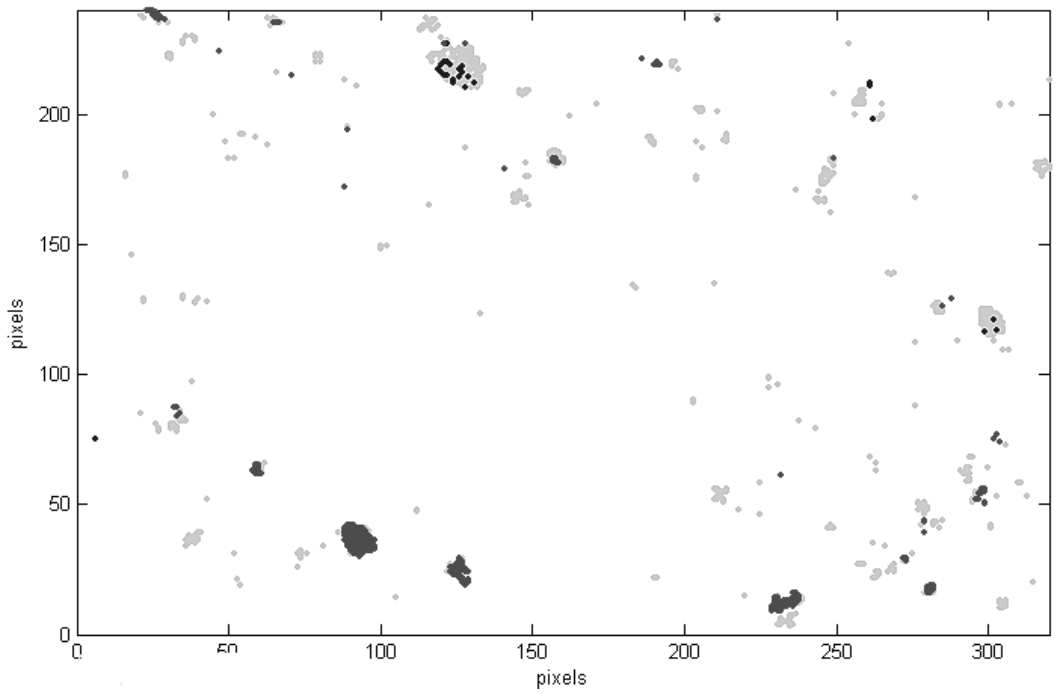


Figure 3. Example of a result obtained with the procedure with the results generated after applying the SVM discrimination model. In black: pixels detected as containing processed animal proteins by the SVM model. In grey: pixels with a maximum peak of absorbance at 1500 nm and absorbance higher than 0.1.

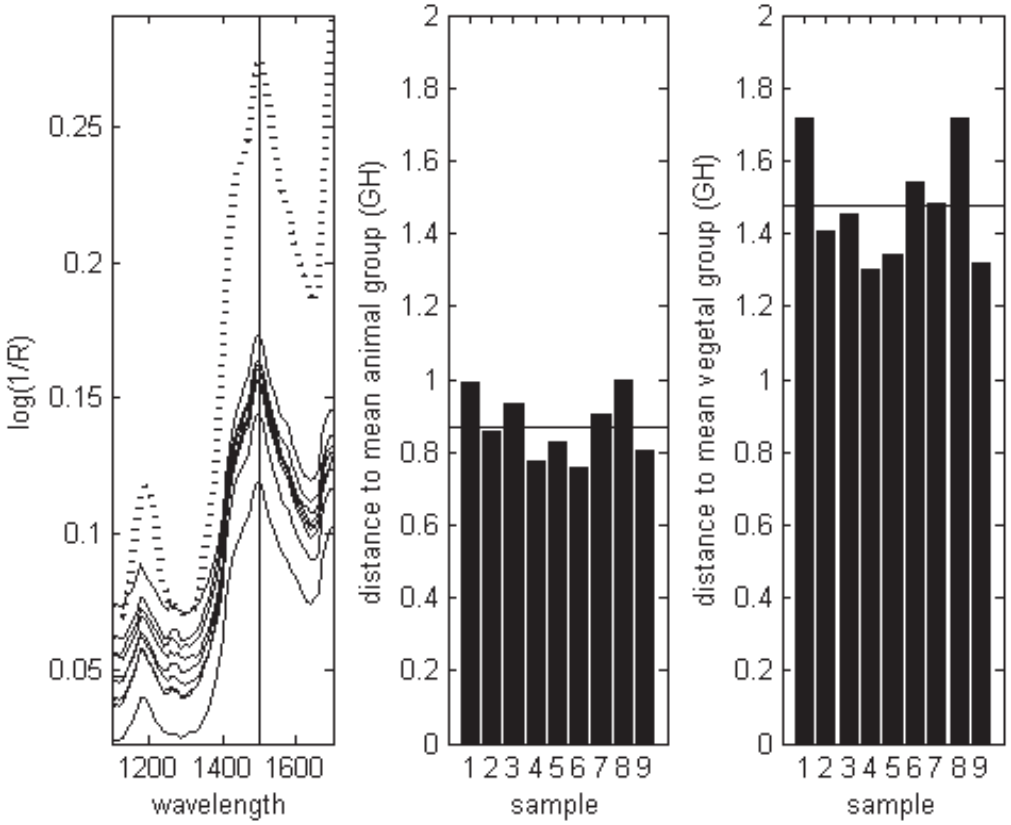


**Figure 4. Visual confirmation of the results. The first plot shows the raw spectra of the selected pixels including, a reference spectrum for pure animal (dashed line). The second and third plots show the Mahalanobis distances for each of the selected spectra to the mean spectrum of the animal group and to the vegetable group, respectively, in the calibration set.**

If there were doubts, the same procedure could be repeated with the smaller fraction of the sample (lower than 250 µm).

This procedure was applied to each image. In total, five images of the raw fraction were taken of each sample and one image of the sediment fraction. The procedure concluded after the application to both fractions.

# Criteria of the validation

In order to validate the method, various criteria and tests were applied to assess LOD, the risk of cross-contamination, the stability and application on independent datasets.

## Determining the limit of detection

In analytical chemistry, the LOD is the lowest quantity of a substance (in this case, MBM) that can be distinguished from the absence of that substance.

The legislation does not permit the presence of any MBM in feedstuffs. An LOD of 0.1% is usually required in order to assess whether the method is suitable for the purpose.[24]

A practical way of determining the LOD is to analyse several samples of different concentrations near to the expected LOD. In this study, tests were performed with samples of feedstuffs contaminated with 0%, 0.1%, 0.3% and 0.5% MBM. For each level of contamination, two measurements were performed (except for 0.3% due to sample availability).

The results presented in Figure 5 show that, for feedstuff with a level of MBM contamination as low as 0.1%, it was possible to detect enough pixels (Figure 5(a)) and particles (Figure 5(b)) to conclude that the sample was contaminated. Particles were visually counted after the application of the procedure. Samples with 0% were detected as such.

## Detecting cross-contamination

The ability of the procedure to detect cross-contamination, whether deliberate or accidental, was checked. This is important in order to avoid any false positives in the samples. For this, two samples were selected. The first one contained 5% MBM (A) and the second did not contain any MBM (B). Six sedimentations were performed on 10 g of feed with three sub-samples of these two samples, alternately. The six sediments were then analysed using the usual NIR imaging method, alternately. Table 1 shows that no cross-contamination occurred during this test and that all sample manipulations were performed safely in terms of the risk of transferring particles from one analysis to another.

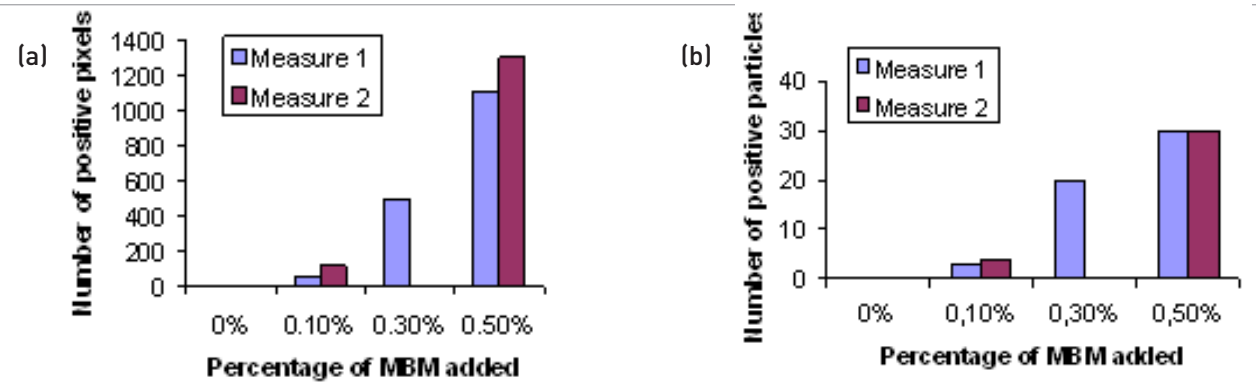


Figure 5. Results of detection by NIR imaging in feedstuffs contaminated with varying percentages of PAP. (a) Results expressed in pixels. (b) Results expressed in particles. Measures 1 and 2 are repeat measurements.

## Stability test (control chart)

Stability can be defined as the ability of a procedure to continue to function, over time and over its full range of uses, without failing or causing failure.

To ensure the quality and reliability of data, it is therefore essential that a procedure is stable through time. This stability can be revealed by plotting a control chart, which is the tool used to determine whether or not a method is under control. A control chart can indicate ongoing performance and, if the performance characteristics of a method have declined, the cause of this should be investigated before proceeding further.[55]

International Standard ISO 17025 (published by the International Organisation for Standardisation) specifies the general requirements for the competence to carry out tests and/or calibrations. Therefore we developed a control chart that tested the ability of the method to give the same results for detection when the analysis was repeated day after day, with some accepted limits. We used a standard sample that was measured every day, and the SVM model was applied to determine the number of pixels detected as containing processed animal proteins. The sample used for the control chart was made of six separated particles consisting of two particles from pure MBM (cattle), two from pure fish and two from plant feed material. From each material, one particle was from the sediment fraction and the other one was from the raw fraction (Figure 6(a)).

The results (number of pixels detected as containing processed animal proteins) after the application of the model were plotted chronologically. This control chart has a central line for the average, two upper lines for the upper control limits and two lower lines for the lower control limits. These lines were determined from historical data. In this study, the control chart was determined using the results of 61 analyses conducted over more than three months (Figure 6(b)). The average value corresponded to the average number of pixels containing processed animal proteins detected by the model; the first limit corresponded to the attention limits or ± two times the standard deviation (SD) and the second limit to the action limits or ± three times the SD.

By comparing new data with these lines, the number of pixels should be within the limits previously determined. In this way, conclusions can be drawn about whether the method is consistent (in control) or is affected by particular causes of variation (out of control). In summary, the control chart determines if the methodology continues to perform as expected and anticipates future problems.

## Results of the validation data sets

In order to test the proposed protocol, samples originating from various inter-laboratory studies were analysed.[48,49]

Table 2 shows the results for 25 negative samples (i.e. samples with 0% MBM) and Table 3 shows the results for 51 positive samples with varying percentages of MBM. In both

Table 1. Results of alternated samples analysis to test if there is cross-contamination.

| Sample | Theoretical percentage | Animal pixels detected | Methodology conclusion |
|---|---|---|---|
| A | 5% MBM | 971 | Positive |
| B | 0% MBM | 0 | Negative |
| A | 5% MBM | 1516 | Positive |
| B | 0% MBM | 0 | Negative |
| A | 5% MBM | 1530 | Positive |
| B | 0% MBM | 0 | Negative |

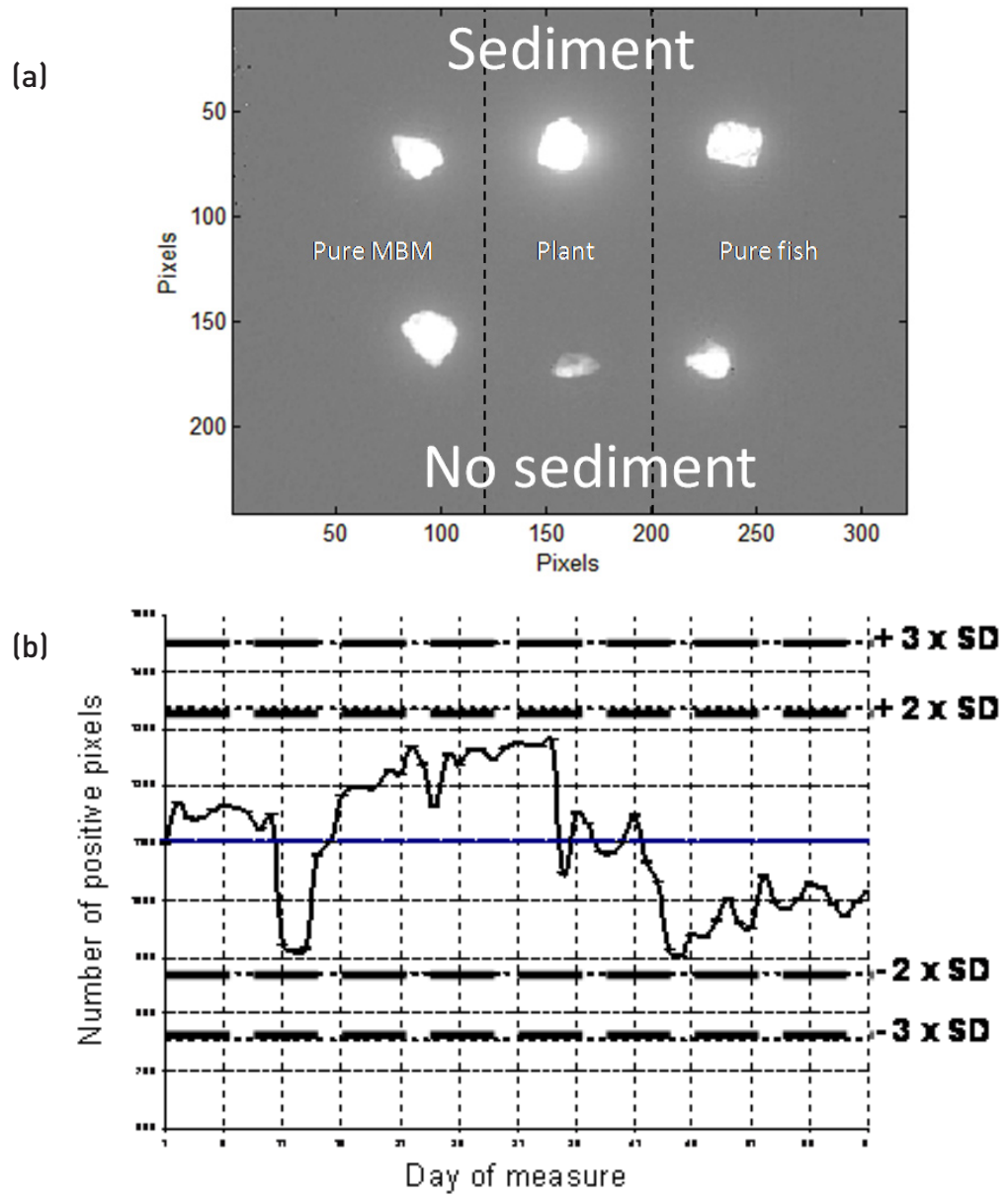


**Figure 6. (a) NIR image of the particles used for the control chart. (b) Control chart showing the range of pixels detected as containing processed animal proteins.**

tables, the source as well as the sample number and the real composition of the samples are indicated. The next column shows the number of pixels detected as positive using the proposed procedure. For each sample, five raw images were taken; this column represents the average number of pixels based on the results of these five images for each sample. The last column gives the final conclusion of the procedure after the application of the SVM equation. This conclusion is based not only on the number of processed animal proteins detected pixels, but also on their distribution using the clustering step as explained in the methodology. If the sample is positive, the pixels cluster together to recreate the shape of a particle, as shown in Figure 3. Samples represented by "+" indicate that they were considered to be positive (i.e. there is a presence of animal particles), whereas samples represented by "–" indicate an absence of animal particles.

When looking at the results in the tables, for some samples the average number of detected pixels was low compared with others with similar MBM percentages, but they were considered to be positive. This is because these pixels grouped together, creating a clear particle. For other samples with a similar number of detected pixels, the conclusion was negative because the pixels did not cluster together and were just individual pixels in the image (false positive results at the pixel level).

From the tables it can be concluded that there was only one false negative result (i.e. a sample that did not seem to

Table 2. Results for 25 negative samples (samples free of meat and bone meal).

| Sample | Source | % MBM real | Animal pixels detected | Conclusion |
|---|---|---|---|---|
| 1 | Stratfeed | 0 | 0 | – |
| 2 | Stratfeed | 0 | 1 | – |
| 3 | Stratfeed | 0 | 0 | – |
| 4 | Stratfeed | 0 | 0 | – |
| 5 | Stratfeed | 0 | 0 | – |
| 6 | Stratfeed | 0 | 7 | – |
| 7 | Stratfeed | 0 | 2 | – |
| 8 | Stratfeed | 0 | 5 | – |
| 9 | Stratfeed | 0 | 2 | – |
| 10 | Stratfeed | 0 | 0 | – |
| 11 | Stratfeed | 0 | 0 | – |
| 12 | Stratfeed | 0 | 0 | – |
| 13 | Stratfeed | 0 | 0 | – |
| 14 | Stratfeed | 0 | 2 | – |
| 15 | Stratfeed | 0 | 1 | – |
| 16 | Stratfeed | 0 | 2 | – |
| 17 | Stratfeed | 0 | 0 | – |
| 18 | Stratfeed | 0 | 6 | – |
| 19 | Stratfeed | 0 | 4 | – |
| 20 | Stratfeed | 0 | 1 | – |
| 21 | Stratfeed | 0 | 0 | – |
| 22 | Stratfeed | 0 | 5 | – |
| 23 | Stratfeed | 0 | 0 | – |
| 24 | VLA | 0 | 7 | – |
| 25 | DGSanco 2004 | 0 | 39 | + |

MBM, meat and bone meal

Table 3. Results for 51 positive samples containing varying percentages of meat and bone meal.

| Sample | Source | % MBM real | Animal pixels detected | Conclusion |
|---|---|---|---|---|
| 26 | Stratfeed | 2 | 111 | + |
| 27 | Stratfeed | 4.5 | 452 | + |
| 28 | Stratfeed | 3.5 | 111 | + |
| 29 | Stratfeed | 4.5 | 182 | + |
| 30 | Stratfeed | 5 | 41 | + |
| 31 | Stratfeed | 2.5 | 699 | + |
| 32 | Stratfeed | 1.5 | 71 | + |
| 33 | Stratfeed | 6 | 3437 | + |
| 34 | Stratfeed | 6.5 | 739 | + |
| 35 | Stratfeed | 7 | 963 | + |
| 36 | Stratfeed | 7 | 1420 | + |
| 37 | Stratfeed | 7.5 | 917 | + |
| 38 | Stratfeed | 3.5 | 338 | + |
| 39 | Stratfeed | 4 | 429 | + |
| 40 | Stratfeed | 4.5 | 1525 | + |

Table 3 (continued). Results for 51 positive samples containing varying percentages of meat and bone meal.

| Sample | Source | % MBM real | Animal pixels detected | Conclusion |
|---|---|---|---|---|
| 41 | Stratfeed | 3.5 | 119 | + |
| 42 | Stratfeed | 6 | 946 | + |
| 43 | Stratfeed | 6.5 | 170 | + |
| 44 | Stratfeed | 5 | 1442 | + |
| 45 | Stratfeed | Pure animal | 89 | + |
| 46 | Stratfeed | 5 poultry | 136 | + |
| 47 | Stratfeed | 5 fish | 567 | + |
| 48 | Stratfeed | 0.1 + 5 fish | 86 | + |
| 49 | Stratfeed | 0.5 + 5 poultry | 123 | + |
| 50 | Stratfeed | 0.1 | 27 | + |
| 51 | Stratfeed | 0.5 + 2.5 poultry + 2.5 fish | 410 | + |
| 52 | IAG | pure fish | 49,932 | + |
| 53 | IAG | fish + 0.1 | 61 | + |
| 54 | IAG | 0.1 | 1 | – |
| 55 | VLA | 0.1 | 81 | + |
| 56 | VLA | 0.5 | 91 | + |
| 57 | VLA | 0.1 + 1 fish | 100 | + |
| 58 | VLA | 0.3 | 109 | + |
| 59 | DGSanco 2003 | 0.1 | 38 | + |
| 60 | DGSanco 2003 | 0.5 | 29 | + |
| 61 | DGSanco 2003 | 0.5 + 5 fish | 67 | + |
| 62 | DGSanco 2003 | 5 fish | 42 | + |
| 63 | DGSanco 2003 | 0.1 + 5 fish | 119 | + |
| 64 | DGSanco 2003 | 0.5 + 5 | 65 | + |
| 65 | DGSanco 2003 | 5 | 57 | + |
| 66 | DGSanco 2004 | 5 fish | 75 | + |
| 67 | DGSanco 2004 | 0.5 feather | 27 | + |
| 68 | DGSanco 2004 | 0.1 | 16 | + |
| 69 | DGSanco 2004 | 0.1 + 5 feather | 456 | + |
| 70 | DGSanco 2004 | 0.1 + 5 fish | 92 | + |
| 71 | DGSanco 2004 | 0.1 | 29 | + |
| 72 | DGSanco 2004 | 0.1 <133 | 15 | + |
| 73 | DGSanco 2004 | 5 | 992 | + |
| 74 | DGSanco 2004 | 0.5 + 5 feather | 352 | + |
| 75 | DGSanco 2004 | 5 feather | 545 | + |
| 76 | DGSanco 2004 | 0.5 | 227 | + |

MBM, meat and bone meal

belong to a particular class, but in fact does; sample 54 in Table 3), corresponding to less than 2% of the contaminated samples. With regard to the false positive results, there was only one sample (sample 25 in Table 2), which is 4% of the 0% MBM samples. The percentage of false positive and negative results is therefore acceptable for a screening and accredited method.

# Conclusion

This paper presents a framework for developing and validating a NIR hyperspectral imaging method as a standard protocol accepted by regulatory authorities using different steps and important criteria to reach this aim. All the criteria and tests used in this study validated the NIR hyperspectral imaging method for the qualitative detection of PAPs in compound

feed. The LOD was about 0.1% and could be even lower by enhancing the number of particles analysed. The control chart showed that, for the different days of analysis, the results were always within the limits allowed and no cross-contamination was indicated. In addition, the percentage of false positive and negative results was acceptable for a screening and accredited method.

This protocol has been fully tested and validated through different studies in line with the International Standard ISO 17025. So far, there have been very few papers concerning NIR methods running under accreditation and dealing with a fully detailed and validated protocol. For this reason, this work is a valuable additional contribution to novel NIR science. This protocol is essential in order to transfer the method to other laboratories and introduce this technology to official control at the laboratory level.

Future work will involve validating the method through an inter-laboratory study and setting a quantitative method for the possible introduction of certain tolerance levels with regard to small quantities of MBM in animal feed. As explained by the European Food Safety Authority at the Scientific Opinion of the Panel on Biological Hazards on 17 October 2007, in a hypothetical situation in which inter-species recycling is allowed, it is not possible, currently, to quantify the level of contamination with non-authorised products containing PAPs in feed. This is because it is not possible to set a correct limit of quantification, as there are insufficient data on the performance of relevant detection methods.

## Acknowledgements

The authors thank Antoine Michotte-Renier, Théophile Buhigiro, Isabelle Fissiaux, Innocent Gasana and Arnaud Boudinot for their competent cooperation in the measurement and analysis work, as well as their valuable advice. We also thank Ouissam Abbas for helpful suggestions on this manuscript.

## References

1. European Commission. *The TSE (Transmissible Spongiform Encephalopathy) Roadmap*. EC document published on 15 July 2005, 24 pages. Available at: http://europa.eu.int/comm/food/food/biosafety/bse/roadmap_en.pdf (2005).
2. *The BSE Inquiry*. http://www.bse.org.uk/pdf/index.htm (2000).
3. European Commission, "Commission Decision 94/381/EC of 27 June 1994 concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of mammalian derived protein", *Off. J. Eur. Commun*. **L 172,** 7 July, p. 23 (1994).
4. European Commission, "Council Decision 2000/776/EC of 4 December 2000 concerning certain protection measures with regard to bovine spongiform encephalopathy and the feeding of animal protein", *Off. J. Eur. Commun*. **L 306,** 7 December, p. 32 (2000).
5. C.L. Lasmèzas and D.B. Adams, "Risk analysis of prion disease in animals", *Sci. Tech. Rev.* **22(1),** 344 (2003).
6. O. Fumière, P. Veys, A. Boix, C. von Holst, V. Baeten and G. Berben, "Methods of detection, species identification and quantification of processed animal proteins in feeding stuffs", *Biotechnol. Agron. Soc. Environ.* **13(S),** 59 (2009).
7. H.J.M. Aarts, E.M. Bouw, J.B. Buntjer, J.A. Lenstra and L.W.D. van Raamsdonk, "Detection of bovine meat and bone meal in animal feed at a level of 0.1%", *J. AOAC Int*. **89(6),** 1443 (2006).
8. O. Fumière, M. Dubois, V. Baeten, C. von Holst and G. Berben, "Effective PCR detection of animal species in highly processed animal by-products and compound feeds", *Anal. Bioanal. Chem.* **385,** 1045 (2006). doi: 10.1007/s00216-006-0533-z
9. M. Prado, G. Berben, O. Fumière, G. van Duijn, J. Mensinga-Kruize, S. Reaney, A. Boix and C. von Holst, "Detection of ruminant meat and bone meals in animal feed by real-time polymerase chain reaction: result of an interlaboratory study", *J. Agric. Food Chem*. **55,** 7495 (2007). doi: 10.1021/jf0707583
10. M. Ansfield, "Production of a sensitive immunoassay for detection of ruminant proteins in rendered animal material heated to > 130°C", *Food Agric. Immunol*. **6,** 419 (1994). doi: 10.1080/09540109409354854
11. M. Ansfield, S.D. Reaney and R. Jackman, "Performance assessment and validation of a sensitive immunoassay for detection of ruminant and porcine heat stable proteins in compound animal feedstuffs", *Food Agric. Immunol*. **12,** 285 (2000). doi: 10.1080/09540100020008155
12. M.C. Aristoy and F. Toldrá, "Histidine dipeptides HPLC-based test for the detection of mammalian origin proteins in feeds for ruminants", *Meat Sci*. **67(2),** 211 (2004). doi: 10.1016/j.meatsci.2003.10.008
13. G. Gizzi, C. von Holst, V. Baeten, G. Berben and L.W.D. van Raamsdonk, "Determination of processed animal proteins, including meat and bone meal, in animal feed", *J. AOAC Int*. **87(6),** 1334 (2004).
14. V. Baeten and P. Dardenne, "The contribution of near infrared spectroscopy to the fight against the mad cow epidemic", *NIR news* **12(6),** 12 (2001).
15. A. Garrido Varo, D. Pérez Marin, A. Gómez Cabrera, J.E. Guerrero, F. De Paz and N. Delgado, "Near infrared spectroscopy as an essential tool in food safety programmes: predicting ingredients in commercial compound feed", in *NIR Spectroscopy: Proceedings of the 10th International Conference*, Ed by A.M.C. Davies and R.K. Cho. NIR Publications, Chichester, UK, p. 145 (2002).
16. I. Murray, D. Perez Marin, A. Garrido Varo, J.E. Guerrero, A. Puigdomenech, P. Dardenne, V. Baeten and J. Zegers, "STRATFEED near infrared instrument network for detecting animal tissues in feedingstuffs", in *NIR Spectroscopy: Proceedings of the 11th International*

*Conference*,Cordoba, *Spain*, Ed by A.M.C. Davies and A. Garrido Varo. NIR Publications, Chichester, UK (2003).

**17.** M.J. De la Haba, A. Garrido-Varo, D.C. Pérez-Marín and J.E. Guerrero, "Near infrared analysis as a first-line screening technique for identifying animal species in rendered animal by-product meals", *J. Near Infrared Spectrosc.* **15,** 237 (2007). doi: 10.1255/jnirs.735

**18.** R.E. Lyons, E. Lesieur, M. Kim, D.C.C. Wong, M.G. Huson, K.M. Nairn, A.G. Brownlee, R.D. Pearson and C.M. Elvin, "Design and facile production of recombinant resilin-like polypeptides: gene construction and a rapid protein purification method", *Protein Eng. Design Selection* **20(1),** 25 (2007). doi: 10.1093/protein/gzl050

**19.** L.W.D. Van Raamsdonk, C. von Holst, V. Baeten, G. Berben, A. Boix and J. de Jong, "New developments in the detection and identification of processed animal proteins in feeds", *Anim. Feed Sci. Technol*. **133(1/2),** 63 (2007).

**20.** I. Murray, A. Garrido Varo, D. Perez Marin, J.E. Guerrero, V. Baeten, P. Dardenne, S. Termes, J. Zegers and R. Frankhuizen, "Macroscopic near-infrared reflectance spectroscopy (WP5-NIRS)", in *Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedingstuffs*. European Communities, Brussels, Belgium, p. 27 (2005).

**21.** F. Piraux and P. Dardenne, "Feed authentification by near-infrared microscopy", in *Proceedings of the 9th International Conference on Near Infrared Spectroscopy, Verona, Italy*, Ed by A.M.C. Davies and R. Giangiacomo. NIR Publications, Chichester, UK, pp. 535–541 (1999).

**22.** V. Baeten, A. Michotte-Renier, G. Sinnaeve and P. Dardenne, "Analyses of feedingstuffs by near infrared microscopy (NIRM): detection and quantification of meat and bone meal (MBM)", in *Proceedings of the Sixth International Symposium on Food Authenticity and Safety*. FASIS, Nantes, France, pp. 1–11 (2000).

**23.** V. Baeten, A. Michotte-Renier, G. Sinnaeve, A. Garrido-Varo and P. Dardenne, "Analysis of the sediment fraction of feed by near infra-red microscopy (NIRM)", in *NIR Spectroscopy: Proceedings of the 11th International Conference,* Cordoba, Spain, Ed by A.M.C. Davies and A. Garrido Varo. NIR Publications, Chichester, UK (2003).

**24.** V. Baeten, C. von Holst, I. Fissiaux, A. Michotte-Renier, I. Murray and P. Dardenne, "The near infrared microscopic (NIRM) method: combination of the advantages of optical microscopy and near-infrared spectroscopy (WP5-NIRM)", in *Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedingstuffs*. European Communities, Brussels, Belgium, p. 14 (2005).

**25.** V. Baeten, C. von Holst, A. Garrido Varo, J. Vancutsem, A. Michotte Renier and P. Dardenne, "Detection of banned meat and bone meal in feedstuffs by near-infrared microscopic analysis of the dense sediment fraction", *Anal. Bioanal. Chem*. **382,** 149 (2005). doi: 10.1007/s00216-005-3193-5

**26.** B. De la Roza Delgado, A. Soldado, A. Martinez Fernández, F. Vicente, A. Garrido Varo, D. Perez Marin, M.J. de la Haba and J.E. Guerrero Ginel, "Application of near-infrared microscopy (NIRM) for the detection of meat and bone meals in animal feeds: A tool food and feed safety", *Food Chem*. **105,** 1164 (2007). doi: 10.1016/j.foodchem.2007.02.041

**27.** C. von Holst, V. Baeten, A. Boix, B. Slowikowski, J.A. Fernández Pierna, S. Tirendi and P. Dardenne, "Transferability study of a near-infrared microscopic method for the detection of banned meat and bone meal in feedingstuffs", *Anal. Bioanal. Chem*. **392,** 313 (2008). doi: 10.1007/s00216-008-2232-4

**28.** M.J. De la Haba, J.A. Fernández Pierna, O. Fumière, A. Garrido-Varo, J.E. Guerrero, D. Pérez-Marín, P. Dardenne and V. Baeten, "Discrimination of fish bones from other animal bones in the sedimented fraction of compound feeds ny near infrared microscopy (NIRM)", *J. Near Infrared Spectrosc*. **15,** 81 (2007). doi: 10.1255/jnirs.688

**29.** A. Pacheco, A. Bannari, K. Staenz and H. McNairn, "Deriving percent crop cover over agriculture canopies using hyperspectral remote sensing", *Can. J. Remote Sens*. **34(1),** 110 (2008).

**30.** Y. Li, A. Vodacek, R.L. Kremens, A.E. Ononye and C. Tang, "A hybrid contextual approach to wildland fire detection using multispectral imagery", *IEEE Trans. Geosci. Remote Sens*. **43,** 2115 (2005). doi: 10.1109/TGRS.2005.853935

**31.** K. Staenz, R.A. Neville, J. Lévesque, T. Szeredi, V. Singhroy, G.A. Borstad and P. Hauff, "Evaluation of casi and SFSI hyperspectral data for environmental and geological applications—two case studies", *Can. J. Remote Sens*. **25(3),** 311 (1999).

**32.** P. Colarusso, L.H. Kidder, I.W. Lewin, J.C. Fraser, J.F. Arens and E.N. Lewis, "Infrared spectroscopy imaging: from planetary to cellular systems", *Appl. Spectrosc*. **52(3),** 106A (1998). doi: 10.1366/0003702981943545

**33.** L.A. Hindus, "IR: the hottest technology in military imaging", *Adv. Imag*. **17(4),** 28 (2002).

**34.** V. Baeten, J.A. Fernández Pierna, A. Michotte-Renier and P. Dardenne, "Imagerie infrarouge : analyse de l'alimentation animale", *Techniques de l'Ingénieur* **3RE34,** 1 (2005).

**35.** V. Baeten and P. Dardenne, "Applications of near-infrared imaging for monitoring agricultural food and feed products", in *Spectrochemical Analysis Using Infrared Multichannel Detectors*, Ed by R. Bhargava and I. Levin. Blackwell Publishing Ltd, Oxford, UK, p. 283 (2005). doi: 10.1002/9780470988541.ch13

**36.** V. Baeten, J.A. Fernández Pierna and P. Dardenne, "Hyperspectral imaging techniques: an attractive solution for the analysis of biological and agricultural materials", in *Techniques and Applications of Hyperspectral Image Analysis*, Ed by H.F. Grahn and P. Geladi. John Wiley & Sons Ltd, Chichester, UK, p. 289 (2007). doi: 10.1002/9780470010884.ch12

**37.** J.A. Fernández Pierna, V. Baeten, J. Dubois, J. Burger, E.N. Lewis and P. Dardenne, "NIR imaging—theory and

applications", in *Comprehensive Chemometrics*, Ed by S. Brown, R. Tauler and B. Walczak. Elsevier, Amsterdam, Netherlands, p. 173 (2009).

**38.** D. Bertrand and E. Dufour E, *La spectrométrie infrarouge et ses applications analytiques*. TEC&DOC, Lavoisier, Paris, France (2006).

**39.** R.C. Lyon, D.S. Lester, E.N. Lewis, E. Lee, L.X. Yu, E.H. Jefferson and A.S. Hussain, "Near-infrared spectral imaging for quality assurance of pharmaceutical products: analysis of tablets to assess powder blend homogeneity", *AAPS PharmSciTech*. **3(3),** article 17 (2002). doi: 10.1208/pt030317

**40.** G. Reich, "Near infrared spectroscopy and imaging: basic principles and pharmaceutical applications", *Adv. Drug Del. Rev*. **57,** 1109 (2005). doi: 10.1016/j.addr.2005.01.020

**41.** P.M. Mehl, Y.R. Chen, M.S. Kim and D.E. Chan, "Development of hyperspectral imaging technique for the detection of apple surface defects and contaminations", *J. Food Eng*. **61,** 67 (2004). doi: 10.1016/S0260-8774(03)00188-2

**42.** J.A. Fernández Pierna, V. Baeten, A. Michotte Renier, R.P. Cogdill and P. Dardenne, "Combination of support vector machines (SVM) and near infrared (NIR) imaging spectroscopy for the detection of meat and bone meat (MBM) in compound feeds", *J. Chemometr*. **18,** 341 (2004). doi: 10.1002/cem.877

**43.** J.A. Fernández Pierna, P. Volery, R. Besson, V. Baeten and P. Dardenne, "Classification of modified starches by FTIR spectroscopy using support vector machines", *J. Food Agric. Chem*. **53(17),** 6581 (2005). doi: 10.1021/jf0501544

**44.** D. Pérez Marín, A. Garrido Varo, J.E. Guerrero and B. de la Roza, "In-house validation of NIRS chemometric models to test the presence of animal origin meals in unground compound feedingstuffs". in *Proceedings of the International Symposium on Food and Feed Safety in the Context of Prion Diseases*, CRA-W, Namur, Belgium, pp. 109–110 (2004).

**45.** V. Baeten, A. Michotte-Renier, C. von Holst, I. Fissiaux, I. Murray and P. Dardenne, "In-house validation of the near infrared microscopy (NIRM) technique for the detection of animal meal in feedingstuffs", in *Proceedings of the International Symposium on Food and Feed Safety in the Context of Prion Diseases*. CRA-W, Namur, Belgium, pp. 121–122 (2004).

**46.** *Stratfeed – Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedingstuffs*. European Communities, Brussels, Belgium (2005). Available at: http://stratfeed.cra.wallonie.be/

**47.** A. Garrido Varo, D. Pérez Marin, J.E. Guerrero, A. Gómez Cabrera, C. von Holst, I. Murray, L.W.D. van Raamsdonk and J. Zegers, "Construction of the STRATFEED sample bank and preparation of sample sets", in *Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedingstuffs*. European Communities, Brussels, Belgium, p. 13 (2005).

**48.** C. von Holst, L.W.D. Van Raamsdonk, V. Baeten, G. Strathmann and A. Boix, "The validation of the microscopic method selected in the Stratfeed project for detecting processed animal proteins", in *Strategies and Methods to Detect and Quantify Mammalian Tissues in Feedingstuffs*. European Communities, Brussels, Belgium, p. 20 (2005).

**49.** G. Gizzi and D.I. Givens, *Variability in Feed Composition and its Impact on Animal Production*. FAO, Rome, Italy, p. 39 (2004).

**50.** E.N. Lewis, L.H. Kidder and E. Lee, "NIR chemical imaging—near infrared spectroscopy on steroids", *NIR news* **16(5),** 3 (2005).

**51.** V.N. Vapnik, *The Nature of Statistical Learning Theory*. Springer, New York (2000).

**52.** N. Cristiani and J. Shawe-Taylor, *An Introduction to Support Vector Machines*. Cambridge University Press, Cambridge, UK (2000).

**53.** H. Chih-Wei, C. Chih-Chung and L. Chih-Jen, Website. http://www.csie.ntu.edu.tw/~cjlin/libsvm (2008).

**54.** M. Daszykowski, B. Walczak and D.L. Massart, "Looking for natural patterns in analytical data. 2. Tracing local density with OPTICS", *J. Chem. Inform. Computer Sci*. **42,** 500 (2002). doi: 10.1021/ci010384s

**55.** A.R.C. Hill and S.L. Reynolds, "Guidelines for in-house validation of analytical methods for pesticide residues in food and animal feeds", *Analyst* **124,** 953 (1999). doi: 10.1039/a900603f