Food Control 43 (2014) 155–162

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Differentiation of meat and bone meal from fishmeal by near-infrared spectroscopy: Extension of scope to defatted samples

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ARTICLE INFO

Article history:
Received 19 December 2013
Received in revised form
14 February 2014
Accepted 3 March 2014

Keywords:
Defatted samples
NIRM
Fat extraction
Meat and bone meal
Fishmeal
Feed safety

ABSTRACT

Current and future legislation regarding the use of processed animal proteins in animal nutrition requires the availability of robust analytical methods that allow for proper implementation of corresponding legal restrictions. Near-infrared microscopy (NIRM) is a spectroscopic method that allows for the differentiation between meat and bone meal and fishmeal and it is assumed that the different content and composition of the fat is one of the factors responsible for the observed differences. Here a study of the NIRM method has been conducted in order to check for the influence of intentionally introduced reduction of the fat content on the capability of the NIRM method to correctly classify defatted samples. This has practical implications, since processed animal proteins may be defatted by solvents under real world conditions. The results confirmed that the scope of the NIRM method could be successfully extended to samples that have been previously extracted with nonpolar solvents. Only after the use of stricter techniques such as extraction with chlorinated solvents or hydrolysis the NIRM method produced a higher portion of wrong classifications. However, since these extraction techniques are not often used under real world conditions, the impact upon the use of the NIRM method in the feed sector for the specific application of the differentiation between meat and bone meal and fishmeal is minor.

1. Introduction

Processed animal proteins (PAPs) are by-products obtained from slaughtered animals and are characterised by a high nutritional value. For this reason PAPs are considered as important feed materials and can be used within the European Union for this purpose provided that specific conditions are fulfilled (Regulation (EC) No 1069/2009). However, in the aftermath of the bovine spongiform encephalopathy (BSE) crisis the use of the vast majority of all PAPs and meat and bone meal in feedingstuffs has been banned within the European Union with minor derogations such as the use of fishmeal for non-ruminants (Regulation (EC) No 999/2001). Whilst the prohibition on the feeding of ruminants with PAPs of whatever origin has permanent character, the corresponding ban on *non-ruminant* PAPs for feed for pigs and poultry was mainly based on the lack of appropriate analytical methodology to determine traces of meat and bone meal in feedingstuffs and the differentiation according to their species origin or group of species. The European Commission (EC) considered the availability of such analytical methods as key prerequisite, in order to consider a reintroduction of PAPs into the feed chain as stated in its second TSE Roadmap (EC, 2010, TSE Roadmap 2). Due to the significant progress in the field of analytical methods (EURL Animal Proteins), the EC issued very recently a Regulation in respect to lifting the ban on *non-ruminant* PAPs in feed for use in aquaculture (Regulation (EU) No 56/2013), getting into force in June 2013. In consequence, these PAPs are now feed materials, thus requiring the feed sector and the control laboratories to apply analytical methods to ensure that the above mentioned provisions are well implemented. In particular, the absence of ruminant PAPs in feed and in other PAPs needs to be scrupulously monitored. Another important aspect concerns the analysis of fishmeal to be sure that it does not contain meat and bone meal.

Various methods exist for the detection of PAPs in feed and feed materials (Jorgensen & Baeten, 2012). In the EU only two methods are allowed within the frame of official control, to confirm the

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http://dx.doi.org/10.1016/j.foodcont.2014.03.001
0956-7135/

presence or absence of PAPs, namely light microscopy that identifies traces of meat and bone meal, and polymerase chain reaction (PCR) that delivers information on the species origin of the detected meat and bone meal (Regulation (EC) No 152/2009; Regulation (EU) No 51/2013). Other methods are also applied by the feed sector, such as immunoassays (Raamsdonk, Margy, Kaathov, & Bremer, 2012) and near-infrared microscopy (NIRM) (Fumière et al., 2009). Light microscopy and NIRM are based on the same principle, which is the identification of bone particles or tissues in feedingstuffs, either by visual inspection of the particles or by the evaluation of near-infrared spectra taken from these particles. When differentiating PAPs from feed materials of vegetable origin, corresponding spectra may be evaluated by decision rules applied to the measured absorbances at specific wavelengths (Baeten et al., 2005). The similarities among meat and bone meal spectra obtained from different animal species require the application of chemometric methods to achieve differentiation at species level (Garrido Varo et al., 2005). Different studies performed during the last years have demonstrated the powerful characteristics of NIRM for the detection of meat and bone meal in feedingstuffs (Baeten et al., 2005; Boix, Fernández Pierna, von Holst, & Baeten, 2012; De la Haba et al., 2007; Pérez Marín, Fearn, Guerrero, & Garrido Varo, 2009). The specific NIRM method presented in this paper has been successfully validated for the detection of meat and bone meal in feedingstuffs through a collaborative study (Boix et al., 2012; Fernández Pierna, Boix, et al., 2013), confirming equivalent results compared to optical microscopy (Baeten et al., 2005).

The NIR method is also capable of discrimination between meat and bone meal and fishmeal (De la Haba et al., 2007; Murray, Aucott, & Pike, 2001). The result presented in Murray's paper indicated that differences in the protein and in particular the fat composition were crucial for the differentiation by the means of spectroscopy (Murray et al., 2001). In detail, the spectroscopic analysis revealed that the absorbance in the 1720 nm region was much stronger for meat and bone meal than for fishmeal and the authors concluded that this result was due to the higher content of polyunsaturated fatty acids in fishmeal compared to meat and bone meal.

Main factors that influence the fat content in the PAPs are the composition of the raw materials used for the production of these processed products and the conditions of the specific rendering method applied. The primary purpose of the rendering procedure is to sterilise the raw materials and to separate the animal by-products into their main components, which are (i) proteins along with inorganic substances such as phosphorus and (ii) fat. Significant differences exist in respect to the sterilisation temperatures, since mammalian by-products need to be subjected to strict steam sterilisation at 133 °C according to the European legislation (Regulation (EC) No 1069/2009), whereas fishmeal is treated at temperatures below 100 °C (FAO, 1986). There are different technologies of processing animal by-products, but most of them include a cooking step, drying the material, defatting, mainly by mechanical means such as pressing and grinding the PAPs. The order of some of these steps may be inverted. Typically, meat and bone meal and fishmeal still contain fat ranging from 5 to 15% (Ockermann & Hansen, 2000).

Whilst extracting PAPs with solvents is already applied by the rendering industry (Greene, 2010), another publication (Nebel & Mittelbach, 2006) showed that fat which is still present in meat and bone meal could be extracted with n-hexane at technical scale and used as raw material for biodiesel production. In consequence, it can be assumed that the fat content in the meat and bone meal after the extraction is significant lower compared to meat and bone meal, normally obtained from the rendering industry. It would then be important to know, whether NIRM would still be applicable on such samples to differentiate them from fishmeal. This aspect has important practical implications, because the previous treatment (e.g. fat extraction with solvent or not) of PAPs present in a feed samples is most often unknown.

The objective of the present study was to elaborate on the impact of unavoidable variation in fat content of the PAPs on the capability of the NIRM method to distinguish between meat and bone meal and fishmeal. Since the exact composition of the materials and the type of processing the animal by-products underwent is most often unknown, proven robustness of the method against this variation of the fat is an important prerequisite for NIRM to be used to monitor the composition of feed materials under real world conditions. Since the spectroscopic method applied in this study has been actually developed and validated for commercial PAPs containing fat at typical levels as mentioned above, the purpose of the current study was to check for extension of scope of the method to *defatted* samples.

In order to investigate the impact of reduced fat content in the samples on the correct classification of MBM and fishmeal samples, the test material was first extracted by different techniques and then subjected to NIRM analysis.

2. Materials and methods

2.1. Test materials

Mammalian meat and bone meal (MBM) was obtained from an EU commercial rendering plant. The raw materials contained by-products from slaughtered cows and pigs. The processing included a batch sterilisation performed at 133 °C, followed by water removal and fat separation by pressing the dried material. The final step consisted of milling the material. One commercial fishmeal was provided from another EU commercial plant, where the left over from fish processing were also cooked, dried, defatted and milled. In this case the sterilisation took place at atmospheric conditions. In consequence both samples still contained fat typically found in PAPs obtained from the rendering industry. In order to further characterise the test samples used in this study, the fatty acid profile of the MBM and fishmeal was determined using gas chromatography coupled to a flame ionisation detector (FID). The results (data not shown) confirmed the predominant presence of polyunsaturated fatty acids in the fishmeal compared to MBM. Furthermore, the fat content of the samples were determined by applying the Soxhlet method (Regulation (EC) No 152/2009) and obtaining 12% for MBM and 11% for fishmeal.

2.2. Sample treatment

The treatment of the MBM and fishmeal was based on the principle that identical test material was subsequently subjected to three different extraction techniques, characterised by increased extraction efficacy. First the MBM and fishmeal samples were extracted with the Soxhlet method using light petroleum as specified in Commission Regulation (EC) No 152/2009 for the determination of "directly extractable crude oils and fats in feed materials of plant origin" (Regulation (EC) No 152/2009). In short, 10 g of sample were extracted with 150 mL of light petroleum using a Soxhlet apparatus for a time period of 6 h (10 cycles per hour). The remaining material after the extraction is named as *Fraction S*. Since some of the more polar fat components such as phospholipids, could still remain in this fraction, a portion of *Fraction S* was extracted applying the Folch extraction method with minor modifications as described in Cequier Sánchez, Rodríguez, Ravelo, and Zárate (2008). This method is considered much more severe in terms of extraction since it uses chlorinated organic solvents to

extract the more polar fat fraction. In short, 3 g of *Fraction S* were extracted by immersion in 60 mL of a mixture of CH_2Cl_2/MeOH (2:1, v/v) capped in a 250 mL Duran glass, performing agitation for a time period of 2 h. The samples were filtered with a free-fat filter and the solvent was evaporated. The remaining material after the Folch extraction was named as *Fraction F*. In the third step, a portion of this fraction was than subjected to hydrolysis followed by Soxhlet extraction to guarantee the complete absence of fat in the samples after these three consecutive extractions. Here, another method of Commission Regulation (EC) No 152/2009 was applied, which aims at the determination of "total crude oils and fats in feed materials of animal origin". In short, 2.5 g of *Fraction F* were mixed with 100 mL of HCl 3 M and boiled for 1 h. After cooling, filtration and neutralisation, the solid residue was extracted by Soxhlet extraction following the procedure described above. The remaining material after this treatment was named as *Fraction H*.

The samples before the fat extraction (*untreated* samples) and the obtained fractions S, F and H (*treated* samples) were kept at room temperature for further spectroscopic analysis.

2.3. FTNIR-microscopy

In this study a Fourier transformed near-infrared spectrometer from PerkinElmer (Perkin Elmer Spectrum One NTS system, Belgium) equipped with a Perkin Elmer Spotlight microscope was used. This use of the microscope allowed the measurement of spectra from a high number of individual particles present in the test materials, in order to reflect the inherent heterogeneity of processed animal by-products in the obtained statistical model.

The particles of the studied samples were spread on a Spectralon plate (10 mm × 10 mm) and presented to the FTNIR microscope using an aperture size of 50 μm × 50 μm. The spectra (1282–2500 nm with a resolution of 3 nm) were collected by selecting visually individual particles and focussing the infrared beam on each of them. Each spectrum was obtained co-adding 10 scans from the same particle (Baeten et al., 2005). The number of particles measured with NIRM differed between the various materials used in the study: For the untreated test material the number of particles is indicated for set 1 to set 4 in Fig. 1, respectively. For the fractions S, F and H at least 170 particles were measured.

3. Data treatment

In the frame of the statistical analyses, it has to be noticed that each spectrum corresponds to an independent single particle.

The statistical assessment consisted of the following three steps. First, we used partial least squares discriminant analysis (PLS-DA) to establish a model that allows for a separation of fishmeal from MBM and that was calculated from the spectra of the *untreated* samples. In the second step, a cut-off value for this response variable was calculated to classify new particles. In the third step, the developed PLS-DA model was applied on spectra from a different data set of the *untreated* samples and on spectra of the *treated* samples. The predicted response variables were then compared against the cut-off value calculated in the second step, in order to classify them either as fishmeal or MBM. By the means of *t*-statistics the probability of wrong classification was assessed and used as indication of the performance of the method depending the fat content in the test material obtained after specific fat extraction treatments.

For the development of the PLS-DA model, the calculation of the cut-off value and the classification of new particles in the prediction phase, independent sub sets from *untreated* samples were formed as shown in Fig. 1. The use of the different sets is explained later on in this paper.

The PLS-DA model was built and afterwards applied in the prediction phase using the software package Unscrambler X10.2 (Camo-Oslo, Norway). The random number generation was performed with Matlab R2012a (MathWorks, Natick, MA, USA) and analysis of variance (ANOVA) was done with Minitab 16 (Minitab, State College, PA, USA).

3.1. PLS-DA model

The spectral region from 1655 to 2500 nm was used in the statistical assessment of this study, whereas the region from 1282 to 1654 nm was omitted since the spectral information in this range was not significant for the purpose of the study. In total, 259 wavelengths were used to develop the PLS-DA model. Prior to statistical assessment, the spectra were subjected to the following pre-treatments: First Derivate Savitzky–Golay with polynomial order 2 and 3 points for number of smoothing (symmetric kernel), and Standard Normal Variate (SNV).

In the first step, a preliminary PLS-DA model was established and cross validated using the spectra of set 1 of the *untreated* samples comprising of 128 particles as shown in Fig. 1. The response variable was set +1 for fishmeal and −1 for the MBM. This model was then applied on the spectra of set 2 to calculate the cut-off value as explained in the next section. In the last step the final PLS-DA model was established with set 3 which contained 253 particles and was therefore based on a higher spectra

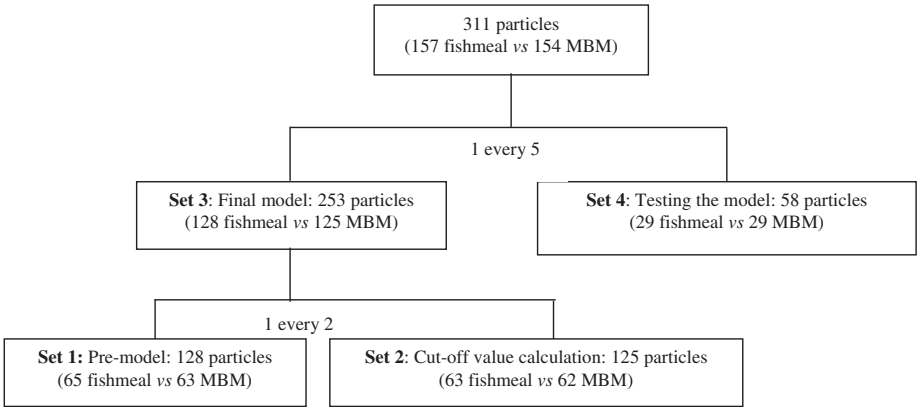

Fig. 1. Distribution of the particles from untreated samples into different sets. From the initial 311 particles, one every fifth particles was selected to build set 4. Per data set it is indicated the target use, the total number of particles and the number of fishmeal and mammalian meat and bone meal particles.

number than the preliminary PLS-DA model. The final model was then used for the prediction of the classification of spectra from *untreated* and *treated* samples, i.e. data set 4 in Fig. 1 and fractions S, F and H. The measurements from five particles had to be removed from the testing set 4, since the corresponding signal intensity was too low.

In the discussion of the results of the multivariate statistics the spectral range and the response variable were abbreviated to *x* and *y* variables, respectively.

3.2. The cut-off value of the response variable

When classifying new particles by applying the PLS-DA model established in the previous section, a cut-off value of the response variable is required against which the measured response value of the new particles is compared. Depending on whether the measured value is above or below the cut-off value, the spectrum is classified as fishmeal or MBM. Considering the chosen dummy variables of +1 for fishmeal and −1 for MBM, the cut-off value was expected to lie between these two values. Instead of setting the cut-off value at 0 without additional measurements, independent measurements from spectra of set 2 were used to establish the cut-off value. We used two criteria for setting the cut-off value, which were to minimise the risk of wrong classification of spectra and to reflect the intrinsic measurement and prediction error of the model including the error linked to the heterogeneity of the samples. The data for calculating the cut-off value were obtained by applying the preliminary PLS-DA model on the independent data set 2 of the *untreated* fishmeal and MBM as specified in Fig. 1. The prediction calculation delivered two results, namely the numerical response for each measurement and the corresponding prediction uncertainty applying the Martens uncertainty test implemented by the software package Unscrambler (Fernández Pierna, Jin, Wahl, Faber, & Massart, 2003). Then the cut-off value was varied across the interval between +1 and −1 and *t*-statistics was applied to estimate the probability that the response value of an MBM particles was *above* of such value or the response value of a fishmeal particles was *below* of such value (Heinrich, Macarthur, von Holst, & Sharman, 2013). In both cases this result would led to a wrong classification of the PAPs concerned. Therefore, these estimations were used to select a specific cut-off value, where the probabilities of wrong classifications for MBM and fishmeal were minimised. This specific cut-off value was then applied to classify new particles.

The following equations were used for the estimation of the probability of wrong classifications, based on the measurements of MBM and fishmeal

$$t\text{-value}_{\mathrm{MBM}} = \frac{\text{cut-off value} - \mathrm{Mean}_{\mathrm{MBM}}}{\mathrm{SD}_{\mathrm{MBM}}} \quad (1)$$

$$t\text{-value}_{\text{fish meal}} = \frac{\mathrm{Mean}_{\text{fish meal}} - \text{cut-off value}}{\mathrm{SD}_{\text{fish meal}}} \quad (2)$$

where "Mean" and "SD" are the means of the predicted response values of the MBM and fishmeal measurements and SD were the corresponding standard deviations respectively. The SD comprised two components, which where the different prediction uncertainties of the measured response variable of each particle and the variation *between* the various measured response variables. ANOVA was applied to calculate the total SD by pooling these two error components. However, the calculation was not straightforward, since the ANOVA algorithm required a set of individual response values, whereas the software package Unscrambler delivered exclusively the corresponding prediction uncertainty. Prior to the ANOVA calculation, Matlab software package was therefore applied to generate 20 random numbers assuming normal distribution and using the measured response variables and the corresponding values for the prediction uncertainty as parameters. For instance, the particle with the measured response variable of 1.1 and a prediction uncertainty of 0.15 was transformed into a vector of 20 numbers distributed between 0.8 and 1.5. In total, 1260 (63*20) numbers were generated for fishmeal and 1240 (62*20) numbers were generated for MBM. These data sets were then subjected to ANOVA to calculate the SD individually for the MBM and the fishmeal required for the determination of the *t*-value according to equations (1) and (2). The probability associated with this *t*-value and using a one-tailed distribution was an estimate of the probability for wrong classification of MBM and fishmeal.

3.3. Probability of wrong classification of new particles

For the classification of new particles, i.e. set 4 of the *untreated* samples and spectra from fraction S, F and H, the same equations already explained in the previous section to estimate the probability of wrong classification have been applied. These equations use (i) the *specific* cut-off value previously selected, (ii) the mean of the response variables obtained when applying the PLS-DA model to the new particles and (iii) the SD, which was the corresponding standard variation of the response values obtained from the individual measurements. The probability associated with this *t*-value using a one-tailed distribution was an estimation of the probability for wrong classification of the PAPs.

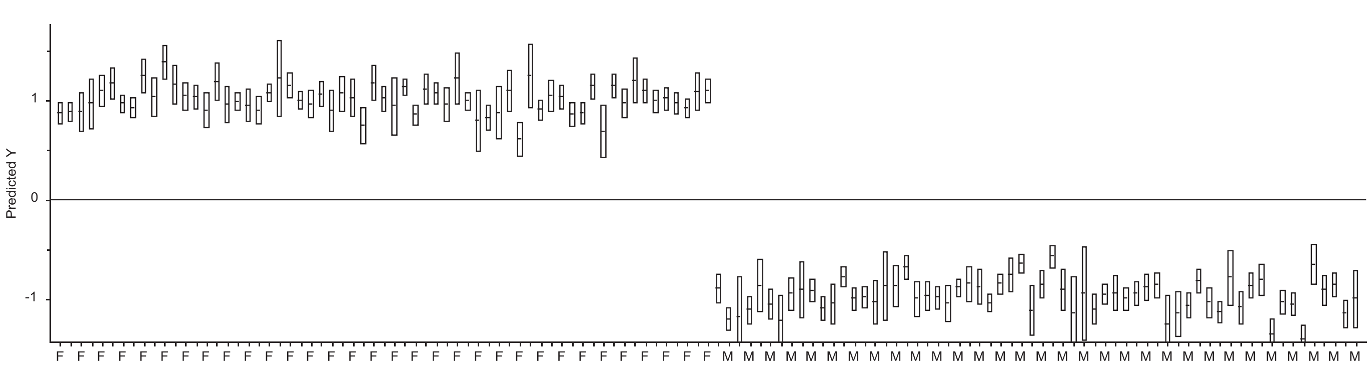

Fig. 2. Preliminary PLS-DA model to establish the cut-off value: The predicted values and the prediction's uncertainty applying the preliminary PLS-DA model on set 2. The response variable was −1 for mammalian meat and bone meal (abbreviated to M) and +1 for fishmeal (abbreviated to F).

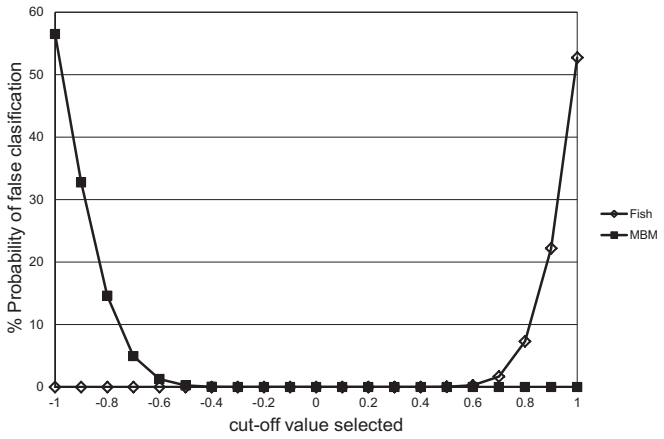

Fig. 3. Selection of the optimal cut-off value using the results from the preliminary PLS-DA model shown in Fig. 2.

The extension of scope was considered acceptable if the correct identification of the meat and bone meal and fishmeal samples was still possible after fat extraction. The benchmark for an acceptable rate of wrong classification was set at 5%, which corresponds to the maximum rate of false compliant results established for some screening methods by European legislation (Commission Decision 2002/657/EC).

4. Results and discussion

4.1. The preliminary PLS-DA model and cut-off values

In Fig. 2 the predicted results for data set 4 obtained with the preliminary PLS-DA model are shown. The figure depicts the predicted response values as vertical bar indicating the prediction's uncertainty. No overlapping between the variables of both groups could be observed, thus indicating that the preliminary model worked well. The predicted response values and the uncertainty prediction were then subjected to ANOVA in order to calculate the probability of wrong classification for different cut-off values as previously described. The outcome of this assessment is shown in Fig. 3, indicating that very low values for the probability of wrong classification were obtained for cut-off values between −0.5 and +0.5. Obviously, cut-off values between −0.5 and −1 would result in a higher rate of wrong classification for MBM. On the other hand, when the cut-off value was set between +0.5 and +1, the rate of wrong classification for fishmeal would be higher. Therefore, zero was taken as cut-off value for the classification of new particles.

4.2. The final PLS-DA model

The classification into the two groups in the calibration and validation phase of the PLS-DA model is presented in Fig. 4, confirming that perfect separation of both groups was obtained. The model was optimised using the first 4 factors, explaining 97% of the total *Y* variance and 89% of the total *X* variance.

In Fig. 5, the wavelengths in the final PLS-DA model that contributed significantly to the separation of both groups are presented. When looking at the two randomly selected spectra prior to the pre-treatment, key spectral bands for the separation could hardly be identified. However, after the pre-treatment by 1st derivative, the spectra revealed some differences between MBM and fishmeal. In particular, the spectral region located in Region 1 between 1712 and 1731 nm which is related to C–H vibrations. In detail, the C–H bonds are shown in NIR spectroscopy due to an asymmetric stretching vibration near 1713 nm, a symmetric stretching vibration near 1754 nm and their combination near

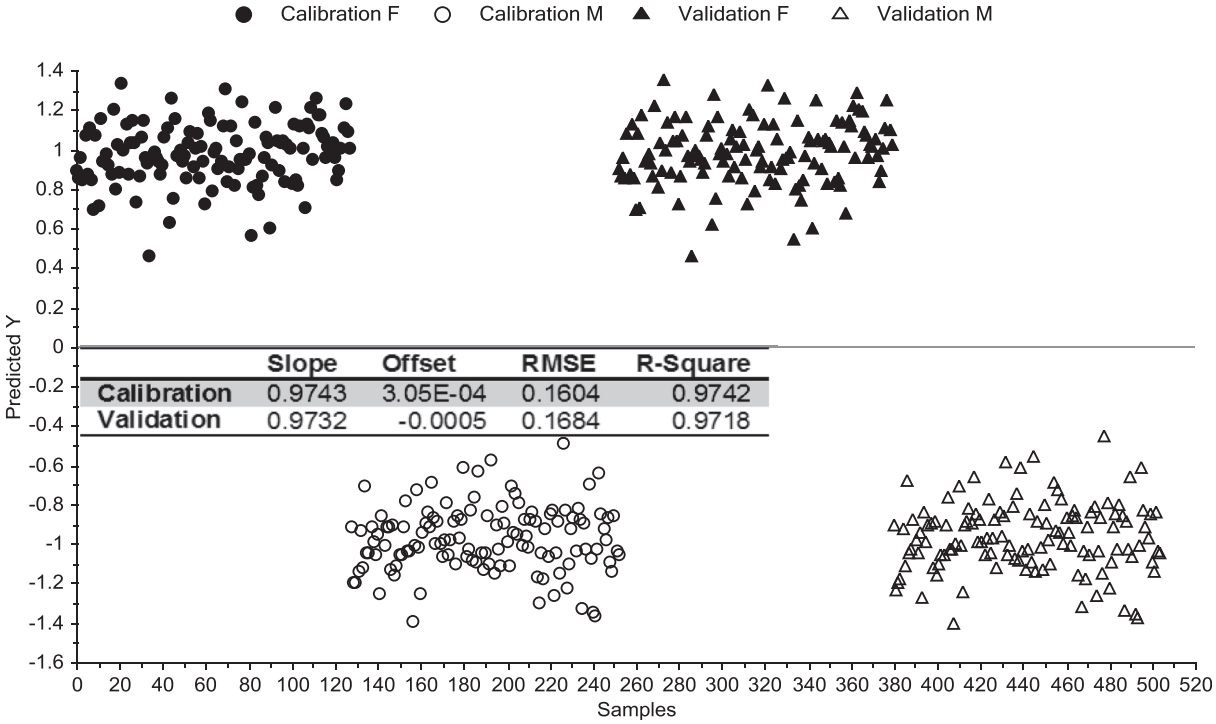

Fig. 4. Performance of final PLS-DA model. Classification of particles in the calibration (circles) and cross validation sets (triangles). The response variable was −1 for mammalian meat and bone meal (abbreviated to M) and +1 for fishmeal (abbreviated to F).

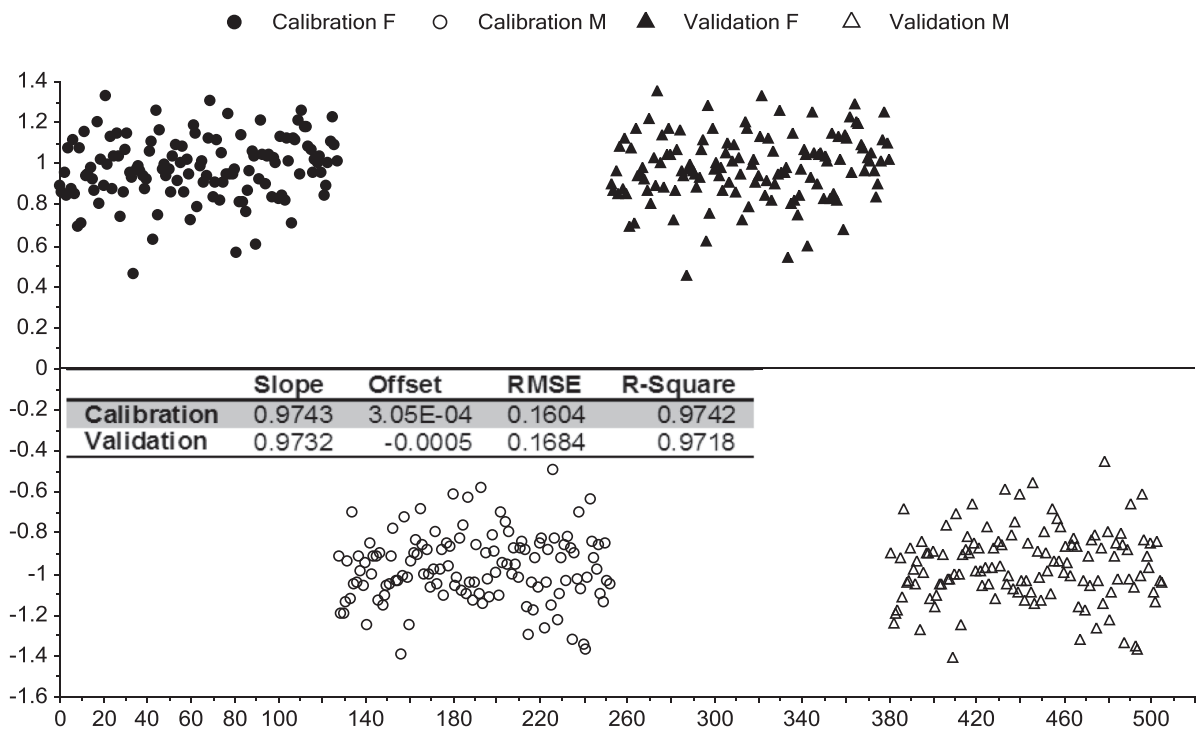

Fig. 5. 1st Derivate spectra of untreated MBM and fishmeal. Wavelengths modelling by the factor 1 and 2 of the final PLS-DA model and its chemical assignation are indicated. **1**: assigned to −CH=CH−, $-CH_3$, $-CH_2-$ structures present in fat; **2**: assigned to RCOH structure presents in proteins; **3**: assigned to −OH, structure presents in H_2O and oxidised compounds; **4**: assigned to $CONH_2$, ROH, ROCH structures presents in proteins; **5**: assigned to $-CH_3$ structure present in fat and proteins.

1733 nm. The maximum of the band in the spectra is located near 1726 nm and maybe produced from the methylene groups ($-CH_2-$) that are more abundant in saturated fat (Baeten, Aparicio, Marigheto, & Wilson, 2000; Hourant, Baeten, Morales, Meurens, & Aparicio, 2000). This aspect may cause the higher intensity of the band in MBM compared to fishmeal. On the other hand the C−H attached to a double bond (C=H) produce the vibration a shorter wavelengths. These results totally agree with the results published by Murray et al. (2001). Other regions of interest were Region 2 assigned to RCOH structure presents in proteins, Region 3 assigned to −OH, structure presents in H_2O and oxidised compounds (Yildiza, Wehling, & Cuppett, 2001) Region 4, assigned to $CONH_2$, ROH, ROCH structures presents in proteins and Region 5, assigned to $-CH_3$ structure present in fat and proteins. In addition, the correlation line loading of the first two factors of the final PLS-DA model indicated that the wavelengths that are modelling these two factors are also responsible for the most significant separation between MBM and fishmeal and are related to functional groups present in monounsaturated and polyunsaturated fatty acids and proteins.

4.3. Performance profile of method applied on untreated and defatted samples

The results of the prediction of the *untreated* and *treated* spectra are presented in Fig. 6. Excellent separation was obtained for the *untreated* samples (Fig. 6a), confirming the results from the preliminary PLS-DA model (Fig. 2). Looking at the prediction of the spectra obtained after the different fat extraction treatments, a very good separation of the vast majority of the particles of fraction S (Fig. 6b) could still be achieved, though the predicted values got closer to each other. For the predictions of the particles of fraction F (Fig. 6c) this trend was reinforced indicating that by further reduction of the fat content the capability of the PLS-DA model to correctly classify PAPs diminished. After the very harsh sample treatment by hydrolysis, both groups cannot be anymore distinguished by using the PLS-DA model as clearly shown in Fig. 6d. It has been considered that this result reflects the efficacy of the treatment to completely extract fat from the samples. In addition, hydrolysis may also change the composition of the protein content, which is probably another factor for the separation of PAPs from both animal groups.

The outcome of the visual inspection of Fig. 6 is corroborated by the estimated rate of wrong classification as summarised in Table 1. Very low values were obtained for the *untreated* samples, since the rate of wrong classification for both groups were below 0.1%. Also the particles of fraction S could be sufficiently separated, since the rate of false classification was <0.1% for fishmeal and 0.54% for MBM and therefore below the target criterion of 5%. In contrast, insufficient separation was obtained for the spectra from fraction F, since these values were fishmeal 5.5% and for MBM 5.7% and therefore above the benchmark of 5%. Finally, after hydrolysis the classification of PAPs seemed to be almost random, since the probability of wrong classification was 9% for fishmeal and 46% for MBM.

The evaluation of the average of the predicted response values as shown in Table 2 revealed that the mean values for fishmeal practically did not change, when comparing *untreated* samples (mean = 0.97) with *treated* samples after Soxhlet extraction (mean = 0.95). In contrast, a distinct decrease of the average values could be observed for MBM, since the mean for the *untreated* samples was −0.98 and for the *treated* samples after Soxhlet extraction was −0.63. This difference may be explained by the higher content of polyunsaturated fatty acids in fishmeal, which are not completely extracted by the means of the Soxhlet method and therefore are still present in the fishmeal. However, after the Folch

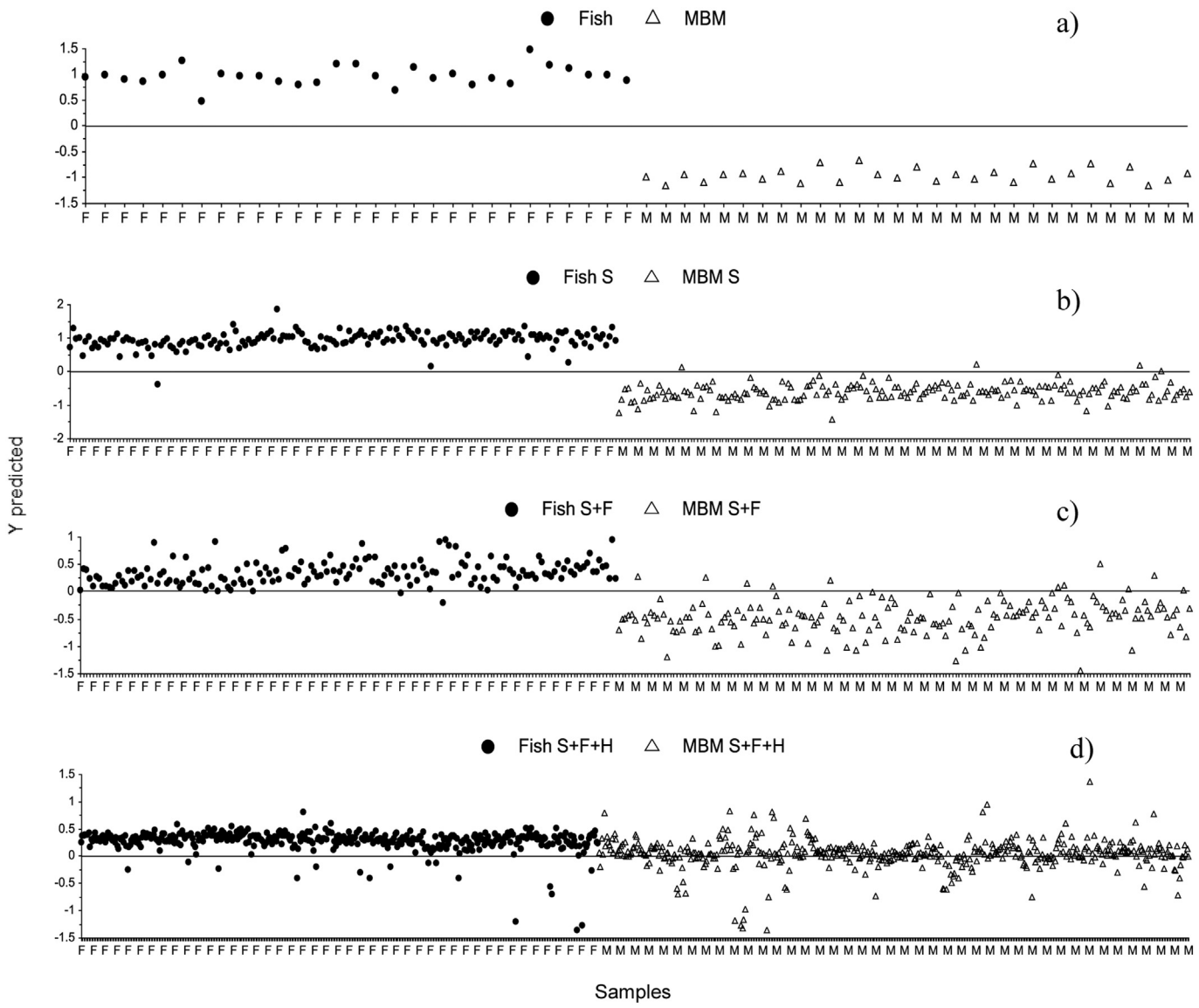

Fig. 6. Predicted values applying the final PLS-DA model to classify MBM and fishmeal: a) Particles from untreated samples; and particles after a sequence of three different fat extractions; b) Soxhlet (S); c) Folch (S + F) and d) Hydrolysis (S + F + H). Results shown with dots are the fishmeal and with triangles are MBM samples. The black line at zero indicates the cut-off value.

extraction the mean value of fishmeal dropped from 0.95 to 0.34, probably due to the major loss of fat in the samples.

The predicted response values were also subjected to ANOVA to estimate the percentage contribution of the *within* and *between* animal group variation to the overall variance observed in the data. It is assumed that the *within* animal group variation reflects the analytical error, whereas the *between* animal group variation includes the factors that cause the differentiation between both groups by NIR. The results of this statistical assessment are presented in Table 2 and demonstrated that for the *untreated* and Soxhlet extraction samples the *between* group variation contributed more than 95% to the overall variation, whereas the impact of

Table 1
Probability of false classification of unknown particles (i.e. MBM as fishmeal or fishmeal as MBM) using zero as cut-off value and applying the final PLS-DA model to (i) untreated samples, and (ii) defatted by Soxhlet (fraction S), by Soxhlet and Folch (fraction F), and by Soxhlet, Folch and hydrolysis (fraction H).

Treatment	Probability (%) of wrong classification	
	Fishmeal as MBM	MBM as fishmeal
None (*untreated* samples)	<0.1	<0.1
Fraction S	<0.1	0.54
Fraction F	5.5	5.7
Fraction H	9	46

Table 2
Mean predicted values per class and per; (i) untreated samples, (ii) defatted by Soxhlet (fraction S), by Soxhlet and Folch (fraction F), and by Soxhlet, Folch and hydrolysis (fraction H). In addition, the percentage variance contributions of between and within animal groups are shown.

	Mean fishmeal	Mean MBM	ANOVA: Partition into variance components (%)	
			Between animal groups	*Within* animal groups
Untreated samples	0.97	−0.98	98.6	1.4
Fraction S	0.95	−0.63	95.5	4.5
Fraction F	0.34	−0.49	83.0	17
Fraction H	0.27	0.02	34.0	66

the analytical error was minor, i.e. 1.4 and 4.5%. For fraction F already 17% of the overall variance was due to the analytical error, whereas for fraction H the contribution from the analytical error exceeded even the *between* animal group variation.

The results from the this study demonstrated that the PLS-DA model developed on processed animal proteins as obtained from the rendering industry produced under real world conditions still worked when the samples used in the prediction exercise had been previously extracted by Soxhlet with light petroleum. Extraction of such materials with organic solvents may be relevant in some technical applications and the proven robustness of the PLS-DA model against such changes of the fat content is considered as important confirmation that the NIRM method including the applied multivariate statistics is fit for the intended purpose. When stricter extraction conditions were applied such as extraction with chlorinated organic solvents or after hydrolysis, the portion of wrong classification increased indicating that this NIRM method is not anymore applicable. However, the authors are not aware of current applications of these techniques in the relevant field within the European Union. Therefore the impact influence of the limitations of the NIRM method for practical applications is considered minor.

5. Conclusions

In the present study a near-infrared microscopy method using PLS-DA to differentiate between meat and bone meal and fishmeal has been sued to evaluate the impact of the different fat content in the samples on the capability of the method to correctly classify samples from both animal groups. Different fat content of the samples was achieved by applying different extraction methods. The evaluation of the spectra confirmed the conclusions from former studies that demonstrated that the higher content of polyunsaturated fatty acids in fishmeal compared to meat and bone meal is an important factor for the differentiation of both groups. The evaluation of the results of analysis of samples extracted with different techniques demonstrated that the scope of the NIRM method could be successfully extended to samples treated by Soxhlet extraction with a nonpolar solvent. In contrast, more severe extraction methods led to an increased portion of wrong classifications. Furthermore the results showed that the experimental design as implemented in this study is a useful tool to evaluate the extension of scope of spectroscopic methods.

Acknowledgements

This research was carried out within the FP6 European Project SAFEED-PAP (FOOD-CT-2006-036221).

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