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On-site quality control of processed land animal proteins using a portable micro-electro-mechanical-systems near infrared spectrometer

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This study was carried out using a spectral database comprising 1164 samples of processed land animal proteins provided by a rendering plant located in southern Spain (Seville). A variety of cloning algorithms and strategies were evaluated for the transfer of spectroscopic databases obtained on a FOSS NIRSystems monochromator (FNS), currently in use for at-line NIR analysis in the rendering-industry laboratory, to a MicroPhazir Rx portable micro-electro-mechanical-systems (MEMS)-based NIR spectrometer. Application of the spectral difference by wavelengths algorithm for spectral adjustment using a 10-sample standardisation set enabled standardisation of the two instruments and effective transfer of spectral data: spectral concordance values, as estimated by the *RMS*(c) statistic, were very similar at 10,829 and 10,388 µlog (1/*R*) for the master (MEMS-NIR) and satellite instruments (FNS), respectively. Once the database had been transferred to the master, calibration equations were developed to predict moisture, crude protein, ash, crude fat, fat after hydrolysis, digestible protein, Ca and P, with a view to comparing the predictive ability of these equations with that of equations obtained using the satellite. Equations developed in the two instruments displayed similar accuracy and precision.

Keywords: MEMS-NIR, rendering, standardisation, calibration transfer, processed animal proteins, land animal proteins.

Introduction [AQ1]

Processed animal protein (PAP) is an EU term applied to rendering materials belonging to category 3 (fit for human consumption). These can be derived from animal by-products (ABP; the specific term "land animal protein" (LAP) has recently been introduced[1]) or from fish. PAPs and LAPs are now highly valued by a number of industries and have become a major complete feed ingredient for pets.

A wide range of PAP ingredients are commercially available – including poultry, pork, feathers, bones and blood – and the percentage of these ingredients used in the manufacture of PAPs varies considerably,[2] prompting marked differences in the chemical composition and nutritional value of end products. The chemical parameters most widely used in marketing PAPs are moisture, crude protein and ashes. However, other parameters are also crucial both for the production process and for ensuring the supply of products of the highest nutritional value. Some pet-food and other feed companies require suppliers to provide information regarding fat content, protein digestibility, Ca and P.

Responding first to the BSE crisis and later on to melamine incidents, scientists, official inspection laboratories, the feed industry in general and the pet-food industry in particular

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have made significant efforts to evaluate and implement a wide range of fit-for-purpose analytical methods capable of overcoming the difficulties encountered with traditional wet chemistry methods, with a view to applying them to a significant volume of samples circulating within and beyond the European market. NIR spectroscopy is considered among the preferred techniques for both quantitative and qualitative analysis of animal protein by-products and feed mixtures containing them.[3,4] In addition to its key role in any rendering and feed-mill quality-assurance programme, NIR spectroscopy may also provide a superbly effective tool in the fight against what is known as "deliberate and economically motivated adulteration", which, though usually not intended to be harmful, is very costly to the feed industry and to consumers. The message from the scientific community has been heeded by a number of companies in the pet-food sector, which have shown that they can incorporate outputs from NIR R&D projects and scientific publications into their businesses, and in doing so exert industry-wide influence. Thus, for example, a leading pet-food manufacturer has introduced NIR-based quality-control systems in order to ensure that feedstuffs provided by suppliers meet quality specifications. Using NIR spectroscopy, the company can decide whether batches from a given supplier should be accepted or rejected.[5] Several years of joint R + D + I activity involving the University of Cordoba and an Andalusian rendering company (Render Grasas S.L.) have culminated in the implementation of NIR technology *at-line* in the plant laboratory, improving the potential for systematic real-time quality control and ensuring a competitive commercial edge. However, as part of the strategy to adopt technological innovations in this and other rendering plants, it is essential to obtain more detailed scientific knowledge regarding the on-site use of NIR technology for real-time analysis, at different points in the manufacturing process.

NIR instrumentation has developed considerably over the last few years, to the extent that highly portable instruments can now be used for process control and on-site analysis, even in the adverse environmental conditions of rendering and feed plants and delivery points. These handheld analysers include sensors based on micro-electro-mechanical-systems (MEMS)-NIR technology; they are compact, robust and portable, and their high throughput speed (milliseconds)[6] enables real-time analysis to be effected at various stages in the process. The use of MEMS-NIR technology is rapidly becoming more widespread in the agro-food industries; as a result, the amount of published research has grown exponentially, with papers focusing on the use of NIR spectroscopy for analysing a whole range of products, including fruits, compound feedstuffs and pork meat.[7,8]

Compared with the substantial literature addressing other raw materials and feedstuffs, research into NIR calibrations for LAPs is relatively limited[9,10] and tends to focus on using monochromators and ground samples.

A key issue in implementing NIR-based feed process controls is to demonstrate that the large, robust and expensive calibration sets already available for laboratory feed analysis, developed over years at a considerable cost by feed manufacturers, may be used for online analysis. The switch to an NIR-based process should not involve starting the calibration procedure all over again for data collected online, as though nothing had been performed before. Cloning or standardisation of instruments, and the transfer of calibrations between instruments, enables full use to be made of the calibration work performed on one instrument when an industry or laboratory acquires a new instrument.[11,12]

Although most published research on standardisation focuses on the use of very similar or identical instruments, attempts have been made more recently to clone rather different instruments.[8,11,13] This research is required because manufacturers and suppliers of NIR instruments tend to provide insufficient information on the real scope for calibration transfer for specific applications/products (e.g. LAPs); and reliable, scientifically rigorous data are essential before a factory commits itself to investing in a brand new instrument.

The primary aim of this study was to transfer a spectral database of LAPs, collected over the last few years using a laboratory-based NIR monochromator, to a handheld MEMS-NIR spectrometer. For this purpose, a number of cloning algorithms and various strategies for generating a standardisation matrix were evaluated. The secondary aim was to develop new calibration equations for LAP chemical composition and other nutritional parameters of interest, using the spectral database transferred to the MEMS-NIR instrument.

Material and methods

Instrument cloning

Samples used and spectral analysis

For standardisation purposes, 25 LAP samples considered representative of a spectral database generated over the last few years (2006–2012) were analysed in their original form (gross milling >4 mm sieve). The "cloning set" comprised 10 samples, while the remaining 15 samples made up the "cloning validation set". Samples were selected with a view to covering the whole range of spectral variability and product absorbance values, using the SELECT algorithm included in the WinISI II version 1.50 software package (Infrasoft International, Port Matilda, PA).

NIR analysis of the 25 samples was carried out simultaneously on a portable MEMS-NIR MicroPhazir spectrometer (Polychromix, Wilmington, MA) (MP; master instrument) and on a FOSS NIRSystems 5000 SY-II monochromator (FOSS NIRSystems, Silver Spring, MD) (FNS; satellite instrument). The MP scans at a non-constant interval of around 8 nm (pixel resolution 8 nm, optical resolution 12 nm), across the NIR wavelength range of 1600–2400 nm, with a scan time per sample of 3 s; the FNS allows measurement from 1100 nm to 2500 nm, with a resolution of 2 nm and a scan time per sample of 120–180 s. Both instruments were located in the laboratory of the rendering company Render Grasas S.L., under controlled environmental conditions (temperature 24°C ± 1°C,

relative humidity 50% ± 10%). With the FNS, each sample was analysed in triplicate using a rectangular sample cup (Nature Product Sample Cup IH-0331) 4.7 cm wide, 20 cm long and 4.3 cm deep, using WINISI II software. With the MP, the same three different subsamples were analysed; 10 spectra were obtained for each subsample, from different points on the sample surface, thus yielding 30 spectra per sample. Spectra were recorded using Phazir Data Management System software (Polychromix, Wilmington, MA). All measurements were made in reflectance mode (log 1/*R*).

Cloning

Instead of transferring prediction models generated on the reference instrument – the procedure habitually used in earlier studies[14] – spectral databases generated on the FNS were transferred to the MP, with a view to subsequently developing various prediction models on the portable instrument. The database to be transferred comprised 1164 LAP samples provided by the rendering company and considered representative of output over the period 2006–2012.

Since the two instruments work at different resolutions and over different spectral ranges, these parameters were adjusted using the interpolation function "interp1" included in the MATLAB software package (The Math Works, Natick, MA) to interpolate the FNS wavelength range (1100–2500 nm with a 2 nm interval) into the MP range (1600–2400 nm with a default 8 nm interval).

A key factor in the cloning process is the number of samples used both when selecting a procedure for standardising NIR instruments and when selecting a cloning algorithm.[8,12] Since cloning using numerous samples is a more complex procedure, it is advisable to minimise the number of samples to be analysed in parallel on the two instruments.[8,12] Here, three strategies using different sample groups were tested: (1) 10 samples comprising the "cloning set" (Group 1); (2) average spectrum for the 10 samples comprising the "cloning set" (Group 2); and (3) sample closest to the centre of the population (Group 3). A total of 12 standardisation matrices were evaluated, combining the different sample groups and four cloning algorithms [direct standardisation (DS), piecewise direct standardisation (PDS) – with two different window sizes, 15 (PDS15) and 93 (PDS93) – and spectral difference by wavelengths (SDW)]. Spectral adjustment, noise elimination and application of the SDW standardisation matrix were carried out using the MATLAB 7.6 software package (The Math Works). The DS and PDS algorithms were developed using the PLS Toolbox software package (Eingenvector Research, Manson, WA).

The cloning algorithms applied are summarised below:

- DS and PDS: Both algorithms use linear transformations based on the expression: $x^a = xF + b$,**[AQ3]** where vector x contains the original spectra, vector x^a contains the adjusted spectra, b is the offset vector (1 × q) and F is the transfer matrix. The transformation matrix is calculated using a partial least-squares (PLS) regression model for each wavelength on the two instruments to be standardised. In the case of the PDS algorithm,[15] local multivariate models are computed for each spectral window around a given wavelength, while DS[16] is individual for each wavelength. Here, the window sizes evaluated for PDS algorithms were of 15 and 93 data points.
- SDW: This algorithm is based on the difference, at each wavelength, between the average spectrum of the standardisation set measured on the two instruments.[17]

Results of standardisation were evaluated using the following statistics, for all 15 samples selected for the validation set:

- The *RMS* (c) statistic, used to compare spectra for the same sample obtained on the two instruments, was calculated using the CONTRAST algorithm included in the WINISI program.

$$\sqrt{\frac{\sum_{i=1}^{n} D_1^2 - \frac{\left[\sum_{i=1}^{n}(y_{i\ \text{master}} - y_{i\ \text{satellite}})\right]^2}{n}}{n-1}} \quad (1)$$

where $D_i = y_{i\ \text{master}} - y_{i\ \text{satellite}}$ are the log(1/*R*) values of two spectra of a single sample scanned in master and satellite instruments at a given wavelength.

- Mahalanobis H values were calculated for the statistics global H (GH), i.e. the distance of a given sample from the centre of the population, and neighbour (NH), i.e. the distance of that sample from its nearest neighbours; limits were set at 3 and 0.6 for GH and NH, respectively.[18]

Comparing the predictive ability of two NIR instruments

In order to demonstrate to the rendering company, in a practical manner, the potential loss of predictive ability following transfer of spectral libraries from the FNS currently used in the plant to the MP (which the company was planning to purchase), equations were developed for the prediction of various compositional parameters – moisture (M), crude protein (CP), ash – and other parameters of interest for marketing of LAPs: pepsin digestible protein (DIGP), crude fat (F), fat after hydrolysis (FH), calcium (Ca) and phosphorus (P). The same number of samples was used in the two instruments, once the database had been transferred from the satellite (FNS) to the master (MP).

Samples

As indicated earlier, the transferred LAP spectral database comprising 1164 samples analysed on the FNS monochromator was used to develop calibrations for the prediction of several compositional parameters: moisture (M), CP, ash, DIGP, crude fat (F), FH, calcium (Ca) and phosphorus (P).

Reference analysis

Reference data for the parameters moisture (M), CP, ash, calcium (Ca), phosphorus (P), DIGP, crude fat (F) and FH were measured following the methods laid down in AOAC.[19] Full reference data were not available for all samples, so the final

number of samples used for each parameter was as follows: M = 1015, CP = 723, ASH = 1077, DIGP = 388, F = 661, FH = 145, Ca = 145 and P = 145.

Calibration development and external validation

Prior to developing prediction equations, the structure and spectral variability of the full population (*N* = 1164) were analysed with a view to detecting spectral outliers; the CENTER algorithm included in the WinISI II software package was used for this purpose. This algorithm performs a principal-component analysis and then determines the centre of the spectral population and calculates the standardised Mahalanobis distance (GH) of each sample from that centre. For agroindustrial products, samples for which GH >3 are generally considered spectral outliers.[18,20]

Once outliers had been identified and removed, the calibration and validation sets were established using the SELECT algorithm included in the WinISI II software package, following the procedure recommended by Shenk and Westerhaus,[18] which enables structured population selection on the basis of spectral data alone. A total of 1000 samples were selected for calibration development, while 133 samples were selected for validation purposes.

The modified partial least-squares regression method was used, and cross-validation was applied in order to determine the optimal number of terms and to detect so-called "chemical outliers"; in all cases, four cross-validation groups were established. The spectral region used was from 1100 nm to 2500 nm (every 2 nm) on the FNS and from 1600 nm to 2375 nm (every ≈8 nm) on the MP. Chemical outliers were detected using the Student *t* test to check for differences between reference and predicted values; samples with a *t* value of over 2.5 were considered outliers.[21]

Combined standard normal variate plus Detrend treatments were used for scatter correction.[22] First- and second-derivative treatments were tested: 1.5.5.1, 1.10.5.1, 2.5.5.1 and 2.10.5.1, where the first digit is the number of the derivative, the second is the gap over which the derivative is calculated (expressed in data points), the third is the number of data points in a running average or smoothing, and the fourth is the second smoothing.[23]

The following statistics were used to evaluate and select the best calibration model for each of the study parameters: standard error of calibration (SEC); standard error of cross-validation (*SECV*); R^2_C (coefficient of determination for calibration) and R^2_{CV} (coefficient of determination for cross-validation); ratio of performance to deviation (*RPD*), i.e. the ratio of standard error of performance to standard deviation; and coefficient of variation (*CV*).[24]

Results for external validation were evaluated using the validation protocol recommended by Windham *et al.*[25] and Shenk *et al.*[20] based on the following statistics: standard error of prediction (*SEP*), standard error of prediction corrected for bias [*SEP*(c)], bias, r^2 (coefficient of determination for validation), Global H (GH) and Neighbour H (NH). This statistical process is based on the determination of a known significant error, termed "bias", and an unexplained significant error, termed *SEP*(c). Generally, for calibration groups comprising 100 or more samples, and validation groups containing nine or more samples, the following control limits are assumed: Limit Control *SEP*(c) = 1.30 × SEC; Limit Control bias = ±0.60 × SEC, minimum 0.6 for r^2 and 3.0 and 0.6 for GH and NH, respectively.

Results and discussion

Determination of the useful wavelength range, spectral analysis and interpretation

Taking decisions and/or making recommendations regarding the purchase of an instrument for a given product application is not an easy task from the scientific viewpoint. Evaluation of the specifications provided by manufacturers is clearly crucial, as is the assessment of performance parameters such as noise, wavelength accuracy and detector response; determination of the total useful wavelength range for the instrument in question is also of paramount importance.

The spectrum of (*N* = 25) LAPs considered representative of the production process, transformed using a first-order

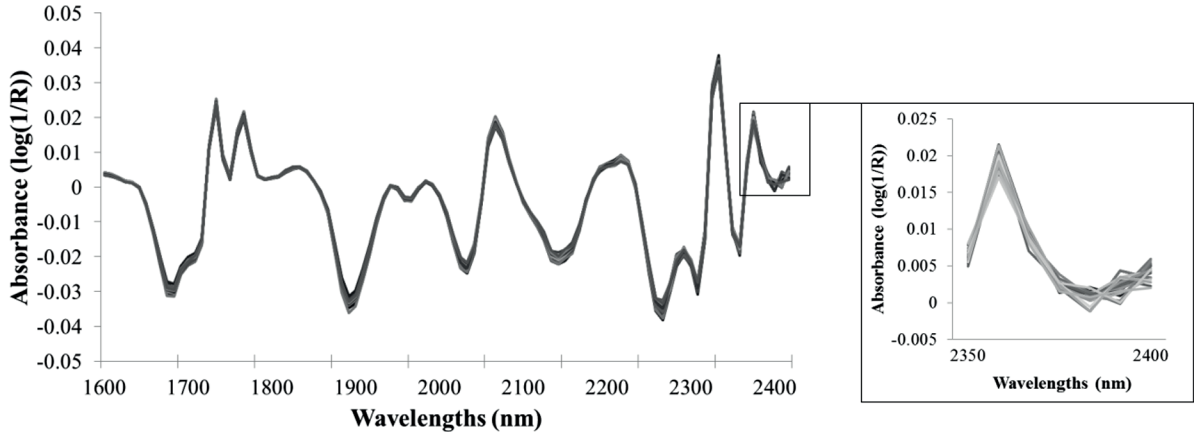

Figure 1. Spectrum after applying first-derivative mathematical treatment: MEMS-NIRS instrument.

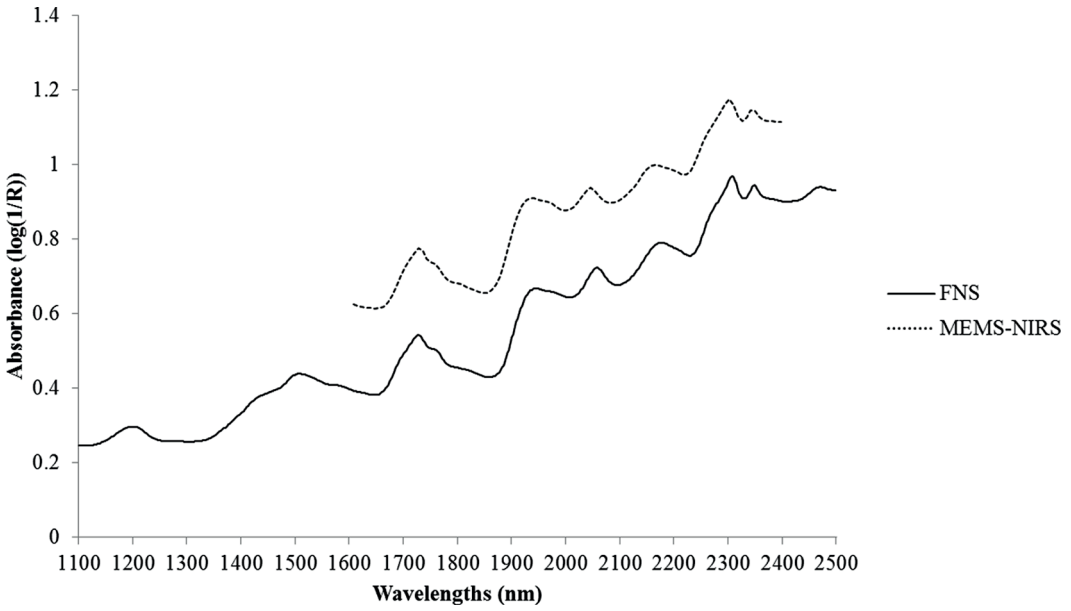

Figure 2. Average NIRS spectra for LAPs obtained on FNS6500 and MEMS-NIRS instruments.

derivative, a single-unit gap and no smoothing (first-derivative treatment 1,1,1,1) recorded in the MP instrument is shown in Figure 1. This type of derivative degrades the signal/noise ratio, so that its application with no additional smoothing leads to the presence in some spectral regions of random oscillations in absorbance values (noise).[26,27] The poor repeatability of the spectra owing to noise (Figure 1) in the wavelength region between 2375 nm and 2400 nm led to this region being eliminated on the MP for subsequent data processing.

Average spectra for LAP samples (N = 1164) obtained using the FNS and the MP are shown in Figure 2. Both instruments yielded similar spectral patterns (absorption peaks aligned on the horizontal axis). However, there were evident differences between spectra with regard to absorbance, attributable to differences between instruments.

The most relevant common absorption bands observed in FNS and MP spectra were located at 1728 nm, 1760 nm, 1940 nm, 2050 nm, 2170 nm, 2304 nm and 2344 nm. Murray[28] was the first to report that the 1212 nm, 1725 nm, 1760 nm and 2308 nm bands were of particular interest in the spectra of organic products, and especially in oils. These bands serve as reference points, since they do not change in similar products. Later on, a number of authors confirmed these findings in different fats and oils.[29] Major absorption peaks in the 1700–1750 nm region for LAPs have been linked to differences in the type of fatty acids. Spectral differences between fishmeal and meat meal are most evident in the 1700–1730 nm region; these are due, at the molecular level, to the higher polyunsaturated fat content in fish than in protein by-product meals. The sharp peak in the 1940 nm band in both spectra is related to

Table 1. RMS(c) values for the "cloning validation set" (N = 15) analysed on the MP and FNS before and after standardisation using various cloning algorithms and sample groups.

Spectral concordance		*RMS* [μlog (1/*R*)]		
		Group 1	Group 2	Group 3
MEMS-NIRS vs.	MEMS-NIRS		10,829	
MEMS-NIRS vs.	FSN**[AQ2]**		88,614	
MEMS-NIRS vs.	FSN Standardised by DS	90,250	82,156	104,836
MEMS-NIRS vs.	FSN Standardised by PDS 15	18,070	82,495	104,836
MEMS-NIRS vs.	FSN Standardised by PDS 93	26,858	82,495	104,836
MEMS-NIRS vs.	FSN Standardised by SDW	10,388	10,388	190,324

Group 1 = 10 samples; Group 2 = Average of 10 samples; Group 3 = Sample closest to centre of population.

Table 2. Mean GH and NH values for the "cloning validation set" (*N* = 15 samples).

Instruments	Mean GH	Mean NH
MEMS-NIRS	2.84	0.85
Unstandardised FNS	4.85	2.18
FNS standardised by SDW	2.80	0.82

water absorption, while peaks visible at 2050 nm and 2170 nm are associated with protein absorption bands.[30–32]

Instrument cloning

Results for the evaluation of standardisation matrices prior to database transfer from the FNS to the MP are shown in Table 1, which lists *RMS*(c) values for each matrix used. Significant differences were observed between instruments prior to standardisation [88,614 µlog (1/*R*)]. The best spectral fit was obtained when applying the SDW algorithm to groups 1 (10 cloning-set samples) and 2 (average spectrum for 10 cloning-set samples), which yielded a fit of 10388 µlog (1/*R*), considered adequate for the standardisation of the two instruments, since a similar spectral concordance was obtained for duplicate samples using the MP [10,829 µlog (1/*R*)].

Other statistics providing information on spectral goodness of fit between instruments are the Mahalanobis global H (GH) and neighbour H (NH) distances. Spectral distances for the validation set using the MP and FNS before and after application of the SDW standardisation matrix are shown in Table 2. The values recorded for the unstandardised FNS (GH = 4.85; NH = 2.18) were higher than those recorded for the MP (GH = 2.84; NH = 0.85) and exceeded the recommended cutoff limits of 3 and 0.6, respectively.[21] After standardisation, however, FNS values for GH and NH (2.80 and 0.82, respectively) lay within recommended limits and were very similar to those obtained using the MP, suggesting that standardisation considerably reduced the main spectral differences.

Calibration development and validation

Having confirmed that application of the SDW standardisation algorithm and subsequent transfer of spectroscopic data yielded acceptable results, the database generated on the FNS was transferred to the MP, applying the best standardisation matrix to all 1164 samples. In practical terms, this meant that the portable MEMS-NIR instrument was now equipped with a large database of samples generated over several years in the rendering plant (2006–2012).

The MP spectral database was treated with the CENTER algorithm, in order to rank spectra in the file according to their H distance (Mahalanobis distance) from the average spectrum. As indicated in the Material and methods section, the CENTER algorithm[18] ranks spectral data on the basis of their standardised H distance from the average spectrum. A total of 31 samples displayed H values of >3.0. Detailed analysis of sample characteristics (chemical and animal species composition) showed that they had "unique features", i.e. high values for M (>7.20%), CP (>80%), F (>20%), FH (>21%) or Ca (>9.0%) and/or were from a single species (100% poultry, 100% pork). These samples were removed from the total set of *N* = 1164 samples, until more similar samples should appear in the manufacturing process at the rendering plant.**[AQ4]** Later on, the SELECT algorithm[18] was applied over the remaining 1133 samples to create calibration and validation sets.

Statistics obtained for compositional values (mean, standard deviation, coefficient of variation and range) in the calibration and validation sets are shown in Table 3. For each parameter, the validation set comprised samples representative of the total variance, all values lying within the range established for the calibration set. The two sets displayed similar values for mean, *SD* and *CV*. As Table 3 shows, the strategy used to select both sets faithfully reflected – using spectral data alone – the variability routinely encountered for LAPs, for each compositional parameter, over the six-year production period studied.

The development of calibrations for practical use by the rendering industry requires a large number of samples representative of the production process. Apart from their value for the calibration development and validation, these large databases enabled the nutritional characterisation of 1133 LAP samples (Table 3). This scientific information is very useful for the rendering industry itself but also for its customers' feed-formulation departments. Detailed knowledge of variations in the composition of batches from any given supplier over a certain period will enable nutritionists to include tighter margins of safety in their nutrient matrices and thus reduce formulation costs.[33] The wide range, high standard deviation

Table 3. Statistics [mean, standard deviation (*SD*), coefficient of variation (*CV*) and range] for calibration and validation sets.

Parameter (%)	Calibration set					Validation set				
	N	Mean	*SD*	*CV* (%)	Range	*N*	Mean	*SD*	*CV* (%)	Range
M	902	2.74	1.25	45.63	0.20–7.20	113	2.74	1.36	49.66	0.60–6.70
CP	641	61.06	6.12	10.02	45.80–76.80	82	62.37	5.95	9.54	50.30–76.60
ASH	953	17.23	4.01	23.28	5.60–30.60	124	16.57	3.72	22.47	8.30–25.80
DIGP	336	91.74	2.51	2.74	82.70–98.10	52	91.76	1.97	2.15	87.20–95.37
F	585	13.25	1.82	13.73	8.50–18.80	76	13.11	1.84	14.05	9.57–17.87
FH	124	14.87	2.37	15.93	9.62–20.45	21	14.38	2.35	16.36	10.96–18.89
Ca	124	4.96	1.73	34.90	1.81–8.70	21	5.08	1.42	28.02	2.90–7.37
P	124	2.45	0.74	30.38	1.01–4.14	21	2.56	0.69	26.96	1.20–3.69

and large coefficient of variation for the compositional parameters shown in Table 3 confirm the considerable variability of the LAPs produced by the rendering company over the 6-year study period. As suggested earlier, this variability is due in part to the production of meals of very different kinds: the very high values for CP (Table 3) reflect the fact that the sample set studied contained not only pork and poultry meals but also blends with a high proportion of feather meals. Alm[34] has noted that feather meals have an average CP value of 80–85%, compared with 60–68% and 45–65% for poultry and pork meals, respectively. The parameters displaying the greatest variability, as estimated by the *CV* in the calibration set, were M (*CV* = 45.63%), Ca (*CV* = 34.90%), P (*CV* = 30.38%), ash (*CV* = 23.28%), and to a lesser extent FH (*CV* = 15.93%) and (*CV* = 13.73%). By contrast, CP (*CV* = 10.02%) and especially DIGP (*CV* = 2.74%) showed less variability.

The results shown in Table 3 generally agreed with those reported by other authors for similar types of meal, although their studies used fewer samples and covered a shorter production period.[35–37] It should be noted that most previous research into the nutritional composition of PAPs focuses on moisture content, CP, crude fat and ash; few studies address levels of calcium, phosphorus, pepsin digestibility or fat by acid hydrolysis, all of which are key parameters for the purchase and sale of PAPs. The Association of American Feed Control Officials[38] states that where feed has been heat treated, or contains calcium salts of fatty acids, the default method for fat determination should be replaced by an acid hydrolysis method; for this reason, FH was included in the present study.

As Table 3 shows, the FH method extracts between 8% and 12% more fat than the traditional method (F). Values for ASH, Ca and P also varied considerably: sales contracts for PAPs tend to specify values for these parameters – and for CP – depending on the species for which the PAPs are intended: thus, poultry and pork meals are commercially available in high-ash and low-ash versions.

Variations in composition similar to those recorded here – though all were produced by the same rendering plant – have also been reported for PAPs produced in other countries. Hendriks *et al.*,[35] in a study of 64 PAP samples obtained from 22 plants over a 2.5-year period, found coefficients of variation comparable with those obtained here for CP (*CV* = 10.3%), F (*CV* =29%) and ASH (*CV* = 25.3%). The authors highlighted the considerable range of interplant and intraplant variation, noting that purchasing of PAPs from the same plant does not necessarily guarantee consistent quality, and that a rapid and inexpensive method for measuring the nutritional quality of PAPs would be highly advantageous. Thus, parameters such as CP – a crucial factor for the customer – tend to vary less than other parameters studied here, and DIGP, though not routinely specified in commercial transactions, tends to display very high mean values (91.74%) and very little variability (*CV* = 2.74%). For rendering plants, consistent values for CP and DIGP play a key role to evidence that the plant is supplying high-quality protein meals.

Calibrations were developed in the 1100–2500 nm region for the FNS and the 1600–2375 nm region for the MP. Statistics for the best NIR calibrations for predicting LAPs chemical composition are shown in Tables 4 and 5. The two instruments

Table 4. Calibration statistics for NIR prediction of chemical composition of animal-based protein meals: FNS instrument.

Parameter (%)	Derivative	*N*	Mean	Range	*SD*	*SECV* (%)	R^2_{CV}	RPD_{CV}	CV_{CV} (%)
M	1,10,5,1	844	2.69	0.20–7.20	1.20	0.48	0.84	2.50	17.89
CP	2,5,5,1	625	60.97	45.80–76.80	6.07	1.72	0.92	3.52	2.83
ASH	1,5,5,1	933	17.25	5.60–30.60	3.99	1.15	0.92	3.48	6.65
DIGP	1,5,5,1	316	91.87	82.63–98.10	2.29	1.32	0.67	1.74	1.44
F	2,10,5,1	556	13.21	8.80–18.60	1.77	0.60	0.88	2.95	4.54
FH	2,10,5,1	120	14.79	10.37–20.45	2.35	0.72	0.90	3.24	4.89
Ca	2,5,5,1	119	4.99	1.81.870	1.71	0.57	0.89	3.03	11.35
P	2,5,5,1	119	2.47	1.01–4.14	0.74	0.29	0.85	2.54	11.78

Table 5. Calibration statistics for NIR prediction of chemical composition of animal-based protein meals: MP instrument.

Parameter (%)	Derivative	*N*	Mean	Range	*SD*	*SECV* (%)	R^2_{CV}	RPD_{CV}	CV_{CV} (%)
M	2,5,5,1	844	2.61	0.20–7.20	1.20	0.52	0.81	2.32	19.87
CP	1,5,5,1	625	60.97	45.80–76.80	6.02	2.11	0.88	2.85	3.47
ASH	2,5,5,1	936	17.20	5.60–30.6	3.95	1.70	0.81	2.32	9.90
DIGP	2,10,5,1	319	91.91	83.61–98.10	2.25	1.47	0.57	1.53	1.60
CF	2,10,5,1	553	13.22	8.80–18.60	1.77	0.62	0.88	2.88	4.66
FH	2,5,5,1	120	14.84	9.62–20.45	2.34	0.77	0.89	3.02	5.21
Ca	1,5,5,1	116	4.92	1.81–8.70	1.73	0.76	0.81	2.29	15.40
P	1,5,5,1	115	2.44	1.04–4.14	0.73	0.39	0.72	1.88	16.00

displayed similar predictive capacity. For all parameters except DIGP, the calibrations accounted for a high percentage of variance (R^2_{CV} > 0.70), although determination coefficient values were always higher in the laboratory FNS instrument (0.92–0.84) than in the portable MP instrument (0.89–0.72). This was to be expected, since the FNS is a monochromator operating over a wider spectral range (1100–2500 nm) than the MP (1600–2400 nm); moreover, the optical window of the FNS sample cup (4.7 cm wide, 20 cm long and 4.3 cm deep) is larger than that of the MP (0.8 cm × 1 cm). Shenk and Westerhaus[39] noted that the goodness of fit of calibrations for which $R^2 \geqslant 0.9$ and R^2 = 0.70–0.89 may be regarded as excellent and good, respectively. Values within this range were recorded here for all study parameters except DIGP, and on both instruments.

As indicated recently by Fearn,[40] while the R^2 statistic can be a useful measure of the performance of a calibration, it does have some limitations. One major constraint is its dependence on the range of values – and on the standard deviation (*SD*) of the reference values – of the calibration set. This would account for the low R^2_{CV} values recorded here for DIGP compared with those of other study parameters, since DIGP displayed the lowest *CV* (2.74%; Table 3). The very slight reduction in the R^2_{CV} (0.67–0.57) for DIGP using the portable instrument (Tables 4 and 5) may be attributable to instrumental differences; the same is true of the modest reduction in the *SD* for the final calibration (once outliers had been removed) using the MP (2.25 vs. 2.29 for the FNS). Fearn[40] has also shown that the *RPD* statistic used in most NIR research is equal to $1/(1 - R^2)$ and depends to the same degree as R^2 on the range of the data in the calibration. This view is borne out by the results obtained here (Tables 4 and 5), which indicate a close match between the highest and lowest *RPD* values in the two instruments and the highest and lowest R^2 values for the respective equations.

The *RPD* statistic is widely used in NIR research for assessing the efficiency of NIR predictions; Williams[24] suggests that *RPD* values of between 3 and 5 indicate acceptable efficiency. If this criterion were applied, only the equations developed here for CP, F, Ca and ASH would be acceptable for routine use. However, in the present authors' view, this criterion cannot be generalised to all types of product or all NIR instruments; it was obtained using equations for NIR analysis of finely ground samples of grains (mainly wheat), using a high-performance laboratory monochromator. Esbensen *et al.*[41] have recently argued that *RPD* values depend on the kind of sample, on its prior preparation, on the way it is presented to the instrument, on the error of the reference method and, in general, on the variance within the sample set used for calibration.

Given the constraints regarding the R^2 and *RPD* statistics, the present paper simply discusses the results with respect to those obtained for similar products (i.e. LAPs) by other authors, taking into account the values recorded for R^2_{CV}, *SECV*, RPD_{CV} and/or CV_{CV}, the two latter statistics either quoted by those authors or calculated from data supplied by them. Use of the CV_{CV} statistic enables correction for differences in the means of the calibration sets used in the various studies examined.

Fontaine and Schirmer[9] developed calibration equations for CP on 333 samples of animal protein meals, reporting values of R^2_{CV} = 0.96, *SECV* = 1.21% and CV_{CV} = 11.6%. Dardenne (personal communication, 2015), in a large collection of LAP samples, excluding feather meals, developed equations to predict M (*N* = 1771), CP (*N* = 2179), F (*N* = 857) and ASH (*N* = 1672), recording values of R^2_{CV} = 0.96, *SECV* = 0.46%, RPD_{CV} = 5.08 and CV_{CV} = 6.05% for M; R^2_{CV} = 0.99, *SECV* = 1.44%, RPD_{CV} = 10.38 and CV_{CV} = 2.52% for CP; R^2_{CV} = 0.97, *SECV* = 0.57%, RPD_{CV} = 6.32 and CV_{CV} = 6.29% for F; and R^2_{CV} = 0.99, *SECV* = 1.21%, RPD_{CV} = 8.53 and CV_{CV} = 6.84% for ASH.

Comparison of these results with those obtained here (Tables 4 and 5) suggests that slight differences in values for the R^2_{CV}, *SECV*, RPD_{CV} statistics with respect to those reported by Fontaine and Schirmer[9] and Dardenne (personal communication, 2015) are largely attributable to small differences in values, means, range and *SD* for the calibration set, and to the fact that both these studies used finely milled samples (0.5–1 mm sieve), whereas the samples used here were as provided by the rendering company (gross milling >4 mm sieve), thus avoiding the laborious fine-grinding process, this being critical for on-site use of the MP instrument. Comparison of the values obtained here for *CV* using the MP (Table 5) and those reported by the two studies indicated earlier shows that values using the MP were lower for the key parameters CP, F and ASH. It should be stressed that the MP instrument costs roughly three times less than the monochromators used by Fontaine and Schirmer[9] and Dardenne (personal communication, 2015).

A review of the literature revealed no references to calibrations obtained for FH, DIGP, Ca or P in LAPs.

The last step in the calibration development process is to validate the equations with an external set of samples. Results for external validation on both instruments using the validation set are shown in Tables 6 and 7. Shenk *et al.*[20] and Windham *et al.*[25] recommend the following control limits for external validation: Limit Control *SEP*(c) = 1.30 × SEC (standard error of calibration); Limit Control bias = ±0.60 × SEC; >0.6 for r^2; and 3.0 and 0.6 for GH and NH, respectively. All the statistics obtained for the equations developed using both the FNS and the MPS (Tables 6 and 7) complied with these recommendations.

The exception was DIGP. The comparatively low r^2 value displayed for DIPG (Tables 6 and 7) may be due to the narrower range and lower *SD* recorded for DIGP compared with M, CP, F, FH, ASH, Ca and P (Tables 4 and 5). This is clearly illustrated in Figure 3, which compares the histograms for CP and DIGP for calibration set samples. For DIGP, most samples lie in the 95% digestibility range, with very little coverage of the range for other DIGP values. By contrast, values for CP are more uniformly distributed over the whole range. In any case, the value obtained for *SEP* (1.63%) is very low compared with values traditionally obtained for digestibility calibrations in other animal feeds. It should be noted that, unlike other compositional parameters such as protein or fat, this

Table 6. Validation statistics for NIR prediction of chemical composition of animal-based protein meals: FNS instrument.

Parameter (%)	*N*	*SEP* (%)	Bias	Bias limit	*SEP* (c) (%)	*SEP* (c) limit	r^2
M	113	0.54	−0.08	±0.29	0.53	0.63	0.85
CP	82	1.95	−0.40	±1.03	1.92	2.24	0.91
ASH	124	1.22	−0.01	±0.69	1.22	1.49	0.90
DIGP	52	1.48	−0.10	±0.79	1.50	1.72	0.39[a]
F	76	0.73	−0.01	±0.36	0.74	0.78	0.84
FH	21	0.76	0.28	±0.43	0.74	0.94	0.91
Ca	21	0.61	−0.14	±0.34	0.62	0.74	0.85
P	21	0.25	0.08	±0.17	0.25	0.38	0.86

Global H = 0.93, Neighbour H = 0.35; [a]exceeding the control limit.

Table 7. Validation statistics for NIR prediction of chemical composition of animal-based protein meals: MEMS-NIRS instrument.

Parameter (%)	*N*	*SEP* (%)	Bias	Bias limit	*SEP* (c) (%)	*SEP* (c) limit	r^2
M	113	0.56	−0.02	±0.31	0.57	0.67	0.82
CP	82	1.86	−0.09	±1.27	1.87	2.75	0.90
ASH	124	1.81	−0.26	±1.02	1.80	2.21	0.77
DIGP	52	1.63	0.00	±0.82	1.64	1.91	0.26[a]
F	76	0.79	0.04	±0.37	0.80	0.80	0.81
FH	21	0.82	0.25	±0.46	0.81	1.01	0.90
Ca	21	0.93	−0.32	±0.45	0.91	0.99	0.78
P	21	0.32	−0.02	±0.23	0.33	0.51	0.78

Global H = 1.06, Neighbour H = 0.14; [a]exceeding the control limit.

parameter cannot be associated with any defined absorption peaks, and considerable interlaboratory and intralaboratory variability exists in the calculation of digestibility parameters.[42] Furthermore, advances in processing methods and equipment have prompted a marked improvement in the digestibility of LAPs in the rendering company concerned, which is known for the high quality and protein digestibility of its LAPs. For that reason, the conventional calibration strategy may not be wholly appropriate in this case, since it would be unrealistic to expect any improvement of the range covered at levels below 95%. Once more spectral data are available for DIGP, it would be useful to evaluate other multivariate analysis strategies based on the detection of spectra that deviate from a given target value. Taking into account the current results for DIGP, this calibration just could be used to detect problems in the production process (i.e. overheating or overpressure as some examples).

Although the loss of performance observed in the validation of equations developed for ash content using the MP as against the FNS is largely attributable to instrumental

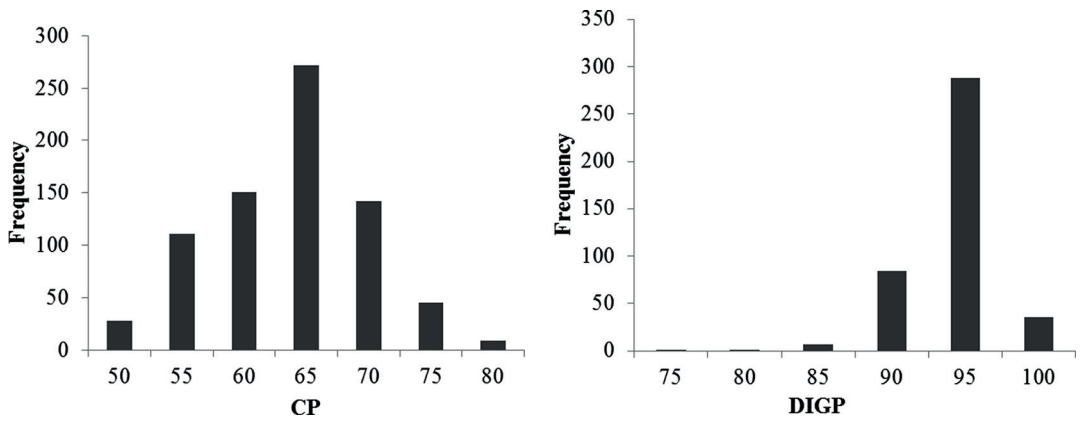

Figure 3. Frequency diagrams for parameters CP and DIGP.

differences, it should also be stressed that ash is by definition not absorbed in the near infrared; published NIR predictions for ash content in a whole range of animal feeds and using a variety of instruments routinely display a poorer predictive capacity than those obtained for parameters such as protein or fat.[42] For these reasons, additional efforts should be made to improve calibrations for ash content, which is crucial for the analytical control of LAPs. Two strategies are currently being evaluated: the first involves a more uniform coverage of the whole range of values for this parameter in the calibration set, using the NH statistic to determine which unknown samples predicted on a daily basis in the plant are under-represented in the calibration set; the second involves the application of nonlinear regression algorithms for calibration purposes, since bone particles present in varying amounts in LAPs may be a major cause of nonlinearity.

Conclusions

The application of the SDW algorithm for spectral adjustment minimised spectral differences between the laboratory FNS monochromator and the handheld MicroPhazir analyser, thus enabling satisfactory cloning/standardisation of equipment. Moreover, the calibration models developed with the database transferred using the best cloning matrix showed that the accuracy and precision of the equations obtained using the handheld instrument were similar, in terms of both calibration and validation, to those of the equations obtained on the FNS monochromator. The potential improvement in the commercial value of LAPs, by fractioning conventional meal proteins into different "quality meals" (i.e. high protein, low ash for aquaculture) is now achievable, thanks to modern equipment. However, innovative technologies such as NIR spectroscopy should also be implemented to control the production process. The handheld MEMS-NIR instrument studied here has been scientifically evaluated to provide the rendering, pet-food and farmed fish industries with valuable information about the advantages of its adoption for *on-site* quality control at the rendering plant and delivery points.

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References

1. S. Woodgate. *Conference Land Animal Proteins: An Opportunity for Aquafeed?* Marine Conservation Society, FABRA, Edinburgh, 9 November 2010 (2010).
2. M.J. De la Haba, A. Garrido-Varo, D.C. Pérez-Marín and J.E. Guerrero, "Near infrared spectroscopy calibrations for quantifying the animal species in processed animal proteins", *J. Near Infrared Spectrosc*. **17,** 109 (2009). doi: http://dx.doi.org/10.1255/jnirs.838
3. I. Murray, L.S. Aucott and H.I. Pike, "Use of discriminant analysis on visible and near infrared reflectance spectra to detect adulteration of fishmeal with meat and bone meal", *J. Near Infrared Spectrosc*. **9,** 297 (2001). doi: http://dx.doi.org/10.1255/jnirs.315
4. A. Garrido-Varo, D.C. Pérez-Marín, J.E. Guerrero, A. Gómez-Cabrera, M.J. De la Haba, J. Bautista, A. Soldado, F. Vicente, A. Martínez, B. De la Roza-Delgado and S. Termes, "Near infrared spectroscopy for enforcement of European legislation concerning the use of animal by-products in animal feeds", *Biotechnol. Agron. Soc. Environ*. **9,** 3 (2005).
5. M. Montoya, J. Laxalde, M. Veleva, J.G. Rosas and F. Soulas, "Control of raw materials with near infrared spectroscopy: a qualitative approach", *NIR News* **24(6)**, 4 (2003). http://dx.doi.org/10.1255/nirn.1385
6. D. Klevisha, "Emerging NIR technologies", presented at the 13th International Conference on Near Infrared Spectroscopy, Umea, Sweden (2007).
7. D. Pérez-Marín, P. Paz, J.E. Guerrero, A. Garrido-Varo and M.T. Sánchez, "Miniature handheld NIR sensor for the on-site non-destructive assessment of post-harvest quality and refrigerated storage behaviour in plums", *J. Food Eng*. **99,** 294 (2010). doi: http://dx.doi.org/10.1016/j.jfoodeng.2010.03.002
8. E. Zamora-Rojas, D.C. Pérez-Marín, E. De Pedro-Sanz, J.E. Guerrero-Ginel and A. Garrido-Varo, "Handheld NIRS analysis for routine meat quality control: Database transfer from at-line instruments", *Chemometr. Intell. Lab. Systems* **114,** 30 (2012). doi: http://dx.doi.org/10.1016/j.chemolab.2012.02.001
9. J. Fontaine and B. Schirmer, "Near-infrared reflectance spectroscopy enables the fast and accurate prediction of essential amino acids contents in soy, rapeseed meal, sunflower meal, peas, fishmeal, meat meal Products and poultry meal", *J. Agric. Food Chem*. **49,** 57 (2001). doi: http://dx.doi.org/10.1021/jf000946s
10. M.J. De la Haba, A. Garrido-Varo, J.E. Guerrero-Ginel and D.C. Pérez-Marín, "Near-infrared reflectance spectroscopy for predicting amino acids content in intact processed animal proteins", *J. Agric. Food Chem*. **54,** 7703 (2006). doi: http://dx.doi.org/10.1021/jf061727v
11. E. Fernández-Ahumada, A. Garrido-Varo, J.E. Guerrero, D.C. Pérez-Marín and T. Fearn, "Taking NIR calibrations of feed compounds from the laboratory to the process: calibration transfer between pre-dispersive and

post-dispersive instruments", *J. Agric. Food Chem.* **56,** 10135 (2008). doi: http://dx.doi.org/10.1021/jf801881n

12. D. Pérez-Marín, A. Garrido-Varo and J.E. Guerrero-Ginel, "Remote near infrared instrument cloning and transfer of calibrations to predict ingredient percentages in intact compound feedstuffs", *J. Near Infrared Spectrosc.* **14,** 81 (2006). doi: http://dx.doi.org/10.1255/jnirs.594
13. M.J. De la Haba, A. Garrido-Varo, N. Núñez, D.C. Pérez-Marín and J.E. Guerrero, "Using calibrations developed for fine milled meat and bone meal on spectra measured on non-milled samples", *J. Near Infrared Spectrosc.* **16,** 275 (2008). doi: http://dx.doi.org/10.1021/jf801881n
14. T. Fearn, "Standardisation and calibration transfer for near infrared instruments: a review", *J. Near Infrared Spectrosc.* **9,** 229 (2001). doi: http://dx.doi.org/10.1255/jnirs.309
15. Y. Wang, M.J. Lysaght, and B.R. Kowalski, "Improvement of multivariate calibration through instrument standardization", *Anal. Chem.* **64,** 562 (1992). doi: http://dx.doi.org/10.1021/ac00029a021
16. Y. Wang, D.J. Veltkamp and B.R. Kowalski, "Multivariate instrument standardization", *Anal. Chem.* **63,** 2750 (1991). doi: http://dx.doi.org/10.1021/ac00023a016
17. J.A. Fernández-Pierna, P. Vermeulen, B. Lecler, V. Baeten and P. Dardenne, "Calibration transfer from dispersive instruments to handheld spectrometers", *Appl. Spectrosc.* **64,** 644 (2010). doi: http://dx.doi.org/10.1016/j.compag.2013.05.007
18. J.S. Shenk and M.O. Westerhaus, "Population structuring of Near Infrared spectra and modified partial least squares regression", *Crop. Sci.* **31,** 1548 (1991). doi: http://dx.doi.org/10.2135/cropsci1991.0011183X003100060034x
19. AOAC. *Official Methods of Analysis of AOAC International*, 18th edition. AOAC International, Gaithersburg, MD (2005).
20. J.S. Shenk, J. Workman and M. Westerhaus, "Application of NIR spectroscopy to agricultural products", in *Handbook of Near Infrared Analysis*, 2nd edition, Ed by D.A. Burns and E.W. Ciurczac. Marcel Dekker, New York, p. 419 (2001).
21. H. Mark and J. Workman, *Statistics in Spectroscopy*. Academic Press, New York (1991).
22. R.J. Barnes, M.S. Dhanoa and S.J. Lister. "Standard Normal Variate transformation and De-trending of near infrared diffuse reflectance spectra", *Appl. Spectrosc.* **43,** 772 (1989). doi: http://dx.doi.org/10.1366/0003702894202201
23. ISI. *The Complete Software Solution Using a Single Screen for Routine Analysis, Robust Calibrations and Networking. Manual, Foss NIRSystems/Tecator*. Infrasoft International, LLC, Silver Spring, MD (2000).
24. P.C. Williams, "Implementation of near-infrared technology", in *Near-Infrared Technology in the Agricultural and Food Industries*, 2nd edition, Ed by P.C. Williams and K. Norris. American Association of Cereal Chemists, St Paul, MN, p. 145 (2001).
25. W.R. Windham, D.R. Martens and F.E. Barton II, "Protocol for NIRS calibration: sample selection and equation development and validation", in *Near Infrared spectroscopy (NIRS): Analysis of Forage Quality. Agriculture Handbook, nº 643*, Ed by G.C. Martens, J.S. Shenk and F.E. Barton II. USDA-ARS, US Government Printing Office, Washington, DC, p. 96 (1989).
26. W.R. Hruschka, "Data analysis: Wavelength selection methods", in *Near-Infrared Technology in the Agricultural and Food Industries*, Ed by P.C. Williams and K.H. Norris. American Association of Cereal Chemists, St Paul, MN, p. 35 (1987).
27. W.F. McClure, "More on derivatives: Part 2. Band shifting and noise", *NIR News* **5(1)**, 12 (1994). doi: http://dx.doi.org/10.1255/nirn.231
28. I. Murray, "The NIR Spectra of homologous series of organic compounds", in *International NIR/NIT Conference*. Akadémiai Kiadó, Budapest, Hungary, p. 13 (1987).
29. A. Garrido-Varo, J. García-Olmo and M.D. Pérez-Marín, "Applications in fats and oils", in *Near-Infrared Spectroscopy in Agriculture,* Ed by C. Roberts, J. Workman and J. Reeves. American Society of Agronomy (ASA), Crop Science Society of America (CSSA) and Soil Science Society of America (SSSA), Madison, WI, p. 487 (2004).
30. I. Murray, L.S. Aucott and H.I. Pike, "Use of discriminant analysis on visible and near infrared reflectance spectra to detect adulteration of fishmeal with meat and bone meal", *J. Near Infrared Spectrosc.* **9,** 297 (2001). doi: http://dx.doi.org/10.1255/jnirs.315
31. D. Cozzolino, A. Chree, I. Murray and J.R. Scaife, "The assessment of the chemical composition of fishmeal by near infrared reflectance spectroscopy", *Aquac. Nutr.*, **8,** 149 (2002). doi: http://dx.doi.org/10.1046/j.1365-2095.2002.00206.x
32. 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-products meals", *J. Near Infrared Spectrosc.* **15,** 237 (2007). doi: http://dx.doi.org/10.1255/jnirs.735
33. W.A. Dozier III, N.M. Dale and C.R. Dove, "Nutrient composition of feed-grade and pet-food-grade poultry by-product meal", *J. Appl. Poult. Res.* **12,** 526 (2003). doi: http://dx.doi.org/10.1093/japr/12.4.526
34. M. Alm. Review of the EU feed ban on non-ruminant Processed Animal Proteins: outlook of the European Fat processors and Renderers Association (EFPRA). TAIEX workshop, Stavropol, 27 November 2012 (2012) http://www.fefac.eu/file.pdf?FileID=42256&CacheMode=Fresh [Accessed: 15 August 2015].
35. W.H. Hendriks, Y.H. Cottam, P.C.H. Morell and D.V. Thomas, "Source of the variation in meat and bone meal

nutritional quality", *Asian-Aust. J. Anim. Sci.* **17,** 94 (2004). doi: http://dx.doi.org/10.5713/ajas.2004.94

36. I.T. Karapanagiotidis, "The Re-Authorization of Non-Ruminant Processed Animal Proteins in European Aqua Feeds", *Fish Aquac. J.* 5 (4), 1 (2014). doi: http://dx.doi.org/10.4172/2150-3508.10000e111

37. R. Nagy and A. Rivis, "Characteristics of meat and bone meal used as animal feed (pet food)", *Studia Universitatis "Vasile Goldiș", Seria Științele Vieții* **24,** 239 (2014).

38. AAFCO. *Fat Best Practices Guidelines*. AAFCO's Laboratory Methods & Services Committee Fat Best Practices Working Group, January 2014. 2014. http://www.aafco.org/Portals/0/SiteContent/Laboratory/Fat_Best_Practices_Working_Group/Fat_Best_Practices_Guidelines.pdf [Accessed 22 August 2015].

39. J.S. Shenk and M.O. Westerhaus, *Routine Operation, Calibration, Development and Network System Management Manual.* NIRSystems, Silver Spring, MD (1995).

40. T. Fearn, "The overuse of R^2", *NIR News*, **25(5)**, 32 (2014). doi: http://dx.doi.org/10.1255/nirn.1464

41. H.K. Esbensen, P. Geladi and A. Larsen, "The *RPD* myth", *NIR News*, **25(5)**, 24 (2014). doi: http://dx.doi.org/10.1255/nirn.1462

42. A. Garrido, "La spectroscopie proche infrarouge: une technologie d´appui pour un service intégral en alimentation animale", in *La spectroscopie infrarouge et ses applications analytiques.* Ed by D. Bertrand and E. Dufour. Collection Sciences & Techniques Agroalimentaires, TEC & DOC, Paris. Chapter 20, p. 473 (2000).

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