Optimisation of the spectral pre-treatments used for Iberian pig fat NIR calibrations

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Abstract

The development of rapid analysis systems for the determination of the fatty acid profile of Iberian pig fat is decisive for the industries involved. Near infrared spectroscopy (NIRS) can fulfil the requirements of this sector, however NIR spectra of fat and oils can be subjected to uncontrolled error sources (changes in the instrument, environment, sample preparation procedure, etc.). Moreover, these errors are particularly important for the calibrations developed for these products, as they present sharp and narrow spectral peaks. There is evidence for correction of the effect of some spectral variations by the use of certain mathematical pre-treatments during calibration. Therefore, this work developed an automatic routine to obtain fatty acid calibrations (palmitic, stearic, oleic and linoleic acid) for Iberian pig fat, using 49 combinations of pre-treatments (first and second derivatives, Auto-Scaling, Detrending and two versions of Multiplicative Scatter Correction), in order to evaluate statistically the effects of different spectral corrections on the prediction ability of NIR equations affected by unexpected variations. Despite that the performance of prediction models showed variations with the mathematical pre-treatment used, generalised significant differences were not detected with the tests applied, except for linoleic acid. However, the optimisation of the derivatives used (order, gap and smoothing segment) proved to be essential for the implementation of robust calibrations.
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1. Introduction

Iberian pig production has become an important sector in the Spanish economy, as their products, particularly dry-cured hams, have acquired an extraordinary reputation at national and international levels due to their high quality [1–3]. Traditional production systems include a final fattening phase called "montanera", which is based on the consumption of natural resources, mainly acorns, in extensive conditions. Different studies have demonstrated that one of the most important factors affecting the quality of Iberian pig products is the composition of the diet during the above cited final feeding process [4]. Particularly, a conventional final feeding in extensive conditions defines the composition and features of sub-cutaneous fat, giving typical profiles for fatty acids and triglycerides composition, as well as the delightful sensorial quality of these products [5,6].

One of the main goals of organizations and industries involved in Iberian pig production is the implementation of an integral quality control system able to certify at any moment the authenticity of products, in order to establish their price as a function of their real quality. Control programs include recording of field data, like live weights at the beginning of "montanera", duration of this phase, amount of available acorns, number of animals, etc., together with laboratory analyses of sub-cutaneous fat. In this sense, the Spanish Ministry of Agriculture, Fishery and Food (MAPA) establishes each year an official norm for the commerce with fattened Iberian pigs destined to slaughtering and transformation, where classification of pigs in commercial categories is set based on quality specifications about animal weights and feeding program; as well as fatty acid contents (palmitic, stearic, oleic and linoleic), determined by gas chromatography in sub-cutaneous fat. As the

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required data for correct classification of products are very expensive, they are only available just for 20 to 50% of total production.

In this context, a new technology based on Near Infrared Spectroscopy (NIRS) arises for rapid analysis of a wide variety of products and parameters. This technology has been involved in an exciting evolution process during the last thirty years, exhibiting nowadays an excellent reputation as an accurate and reproducible technique for qualitative and quantitative analysis in many fields like agro-food, pharmaceutical and chemical industries, human health, environmental studies, etc. There are evidences showing that NIRS technology can fulfil the requirements stated by this sector, as it has been demonstrated in specific works, where there were obtained quantitative models to predict fatty acid contents in pig fat (sub-cutaneous or intramuscular) or qualitative models to classify animals based on their products' NIR spectra [1–3,7–9]. The potential of NIR technology for the prediction of fatty acid profiles has been recently documented for a wide range of products, like vegetable oils [10,11]; fish oil [12]; rabbit and beef meat [13,14]; and egg yolk [15].

Nevertheless, NIR analysis of fats and oils is strongly affected by uncontrolled variations related to the instrument, the environment, the sample preparation protocol, etc. Chemometrics procedures [16,17] are usually applied to NIR spectra in order to extract relevant information, as they are influenced by the above mentioned features and other sources of error [18,19], resulting that precision in NIR analysis is subjected to the availability of algorithms or spectral data pre-treatments developed to reduce these effects. It has been noted that the effect of these unexpected variations in the spectra are particularly important in the fat spectra versus those of other agro-food products, since while the latter generally present very broad absorption bands (more than 200 nm), fats and oils present very sharp and narrow peaks [20].

It has been demonstrated that the sensitivity of calibrations to the unexpected variation sources can be reduced by the use of certain chemometric algorithms like repeatability files [21]. However, the implementation of repeatability files is not an easy task, as it requires a specific design for fats and oils.

On the other hand, the use of one or more spectral pre-treatments during calibration development is a regular practice in the development of NIR prediction models, as some of these transformations reduce significantly the observed residuals (NIR predicted value minus reference value). In this context, it could be interesting to know if these spectral transformations can correct some of the observed unforeseen variations and, as well, determine the degree and extent of the corrections.

While pre-treatments seem to be of importance in calibration development, statistical significance of the improvements obtained in the prediction ability of NIRS equations, after its use, is often not established. Moreover, most published works conclude about the use of a specific pre-treatment on the basis of minimum values obtained for the standard errors of cross validation (SECV) or standard errors of prediction (SEP). This is risky, because any improvement is confounded by other factors, for example outlier rejection.

Recently, several authors [22–25] used statistical tests to compare SEP or bias-corrected SEP (SEP(C)) values obtained after applications of different pre-treatments of NIR spectral data. The statistical tests used are mostly based on a procedure published by Fearn [26]. Conclusions found in these papers are diverse: Roggo et al. [23] indicated that best results for the prediction of sucrose content of sugar beet were achieved with spectra pre-treated with Standard Normal Variate (SNV) and a second derivative; while Delwiche and Reeves [25] concluded that, even with statistical testing, the selection of the appropriate pre-treatment in a NIR calibration must ultimately be based on the modeller's judgment.

Thus, the present paper tries to evaluate the statistical significance of the effect of different mathematical pre-treatments on the sensitivity to unexpected sources of errors, of NIR equations developed for the prediction of main fatty acids in pig fat.

2. Experimental

2.1. Samples and reference data

A total of 341 samples of Iberian pig fat belonging to animals reared during 1997 and 1998 and produced under the guidelines of the Designation of Origin "Jamón de Huelva" were used in this study. Sub-cutaneous fat samples were collected from the tail insertion area in the coxal region of all animals after slaughtering.

Liquid fat samples were obtained after melting in a microwave oven, and were maintained at 35 °C in an oven till they were scanned in a liquid state [27]. Due to the melting procedure used, samples were clear but not totally free of residual moisture or solids.

Table 1
Composition (weight percentage of total methyl esters) of calibration and validation sets

	Entire set				Calibration set				Validation set			
	C16:0	C18:0	C18:1	C18:2	C16:0	C18:0	C18:1	C18:2	C16:0	C18:0	C18:1	C18:2
N	341	341	341	341	311	311	311	311	30	30	30	30
Mean	21.08	10.67	52.32	9.39	21.07	10.64	52.36	9.38	21.20	10.96	51.90	9.45
Min.	17.90	7.70	45.00	6.80	18.00	7.70	45.00	6.80	17.8	7.9	45.8	7.8
Max.	25.30	14.90	58.10	13.50	25.30	14.90	58.10	13.5	24.8	14.20	56.90	13.10
SD	1.46	1.32	2.44	1.32	1.45	1.31	2.43	1.33	1.64	1.46	2.48	1.27

n: number of samples; SD: standard deviation.
C16:0: palmitic acid; C18:0: stearic acid;C18:1: oleic acid; C18:2: linoleic acid.

Fatty acid composition, defined by contents in oleic (C18:1), linoleic (C18:2), palmitic (C16:0) and stearic (C18:0) fatty acids, was determined by gas chromatography on liquid fat, using an HP-5890 chromatograph fitted with automatic injector and an RTX-2330 column.

The entire set was randomly split into a calibration ($n=311$) and a validation set ($n=30$), in order to segregate an independent collective to evaluate the prediction models developed using the remaining samples. Chemical composition data for both sets used is displayed in Table 1.

In order to test the effect of unexpected variations in the spectra, a fat sample was divided into sub-samples that were preserved frozen and scanned after thawing at regular intervals during the spectra recording period.

2.2. NIRS hardware

Reflectance spectra were obtained on a Foss NIRSystems 6500 SY-I monochromator, from 400 to 2498 nm, every 2 nm (spectral bandpass 10 nm±1 nm). Analysis was performed using a spinning module. Samples were scanned using a transflectance cam-lock ring cell (3.75 cm diameter) with 0.1 mm pathlength and provided with an aluminium reflectance surface (FOSS ref. IH-03459). The volume needed per sample was 0.3 ml, approximately. Reflectance data were stored as the logarithm of reciprocal of reflectance ($1/R$), collecting 1050 data points per sample. The spectrum of each sample was the average of the spectra of two fat sub-samples, being each sub-sample spectra the result of 32 successive scans (16–32–16, reference–sample–reference).

2.3. NIRS software

All spectra were manipulated and processed, and all calibration equations were obtained using ISI software NIRS3 ver. 4.0 and WINISI ver. 1.5 (Infrasoft International, State College, PA), respectively.

Statistical tests for SEP(C) comparisons were computed with Microsoft Excel.

2.4. Mathematical pre-treatments

A total of 49 combinations of pre-treatments (Table 2) were applied to obtain Modified Partial Least Squares (MPLS) calibration equations [28,29]. The mathematical pre-treatments used were a spectral Auto-Scaling (AS) based on Standard Normal Variate (SNV) [30], Detrending (DT) [30], combination of AS followed by DT (ADT), combination of DT followed by AS (DTA), two versions of Multiplicative Scatter Correction (MSC) [31] included in the WINISI software (Standard MSC and Weighted MSC) and four different derivative math treatments.

2.4.1. Auto-Scaling (AS) based on Standard Normal Variate (SNV) [30]

The SNV centres each spectrum and then scales it by its own standard deviation (σ), correcting shifts on the log($1/R$) axis.

Table 2
Combinations of pre-treatments used in calibration

Equation	Scatter correction	Derivative	Equation	Scatter correction	Derivative
1	None	None	26	ADT	1,5,5,1
2	AS	None	27	ADT	1,10,5,1
3	DT	None	28	ADT	1,10,10,1
4	ADT	None	29	ADT	2,5,5,1
5	DTA	None	30	ADT	2,10,5,1
6	Standard MSC	None	31	ADT	2,10,10,1
7	Weighted MSC	None	32	DTA	1,5,5,1
8	None	1,5,5,1	33	DTA	1,10,5,1
9	None	1,10,5,1	34	DTA	1,10,10,1
10	None	1,10,10,1	35	DTA	2,5,5,1
11	None	2,5,5,1	36	DTA	2,10,5,1
12	None	2,10,5,1	37	DTA	2,10,10,1
13	None	2,10,10,1	38	Standard MSC	1,5,5,1
14	AS	1,5,5,1	39	Standard MSC	1,10,5,1
15	AS	1,10,5,1	40	Standard MSC	1,10,10,1
16	AS	1,10,10,1	41	Standard MSC	2,5,5,1
17	AS	2,5,5,1	42	Standard MSC	2,10,5,1
18	AS	2,10,5,1	43	Standard MSC	2,10,10,1
19	AS	2,10,10,1	44	Weighted MSC	1,5,5,1
20	DT	1,5,5,1	45	Weighted MSC	1,10,5,1
21	DT	1,10,5,1	46	Weighted MSC	1,10,10,1
22	DT	1,10,10,1	47	Weighted MSC	2,5,5,1
23	DT	2,5,5,1	48	Weighted MSC	2,10,5,1
24	DT	2,10,5,1	49	Weighted MSC	2,10,10,1
25	DT	2,10,10,1			

The transformation is applied to each spectrum individually, and the resulting spectra have mean zero and unit variance.

$$X_{\text{SNV},i} = \frac{X_{\log(1/R),i} - \overline{X}_{\log(1/R)}}{\sigma_{\log(1/R)}}$$

where

$X_{\text{SNV},i}$ is the SNV spectrum value at wavelength i.

$X_{\log(1/R),i}$ is the log($1/R$) spectrum value (raw data) at wavelength i.

$\overline{X}_{\log(1/R),i}$ is the mean value of log($1/R$) spectrum (for all wavelengths).

$\sigma_{\log(1/R)}$ is the standard deviation of log($1/R$) spectrum (for all wavelengths).

i is the wavelength.

The AS algorithm (wrongly called SNV) as included in ISI and WINISI softwares has been simplified excluding spectra centering, so transformed spectra have non-zero mean and unitary variance.

$$X_{\text{AS},i} = \frac{X_{\log(1/R),i}}{\sigma_{\log(1/R)}}$$

2.4.2. Detrending (DT) [30]

DT is performed using a second-order polynomial in a regression analysis, where spectral values of a sample form the response or dependent variable and the independent variable is given by the wavelengths, correcting effects due to baseline curvature. DT correction is also applied to an individual spectrum, and gives a spectrum with zero mean and not-unitary variance.

$$\hat{X}_{\log(1/R),i} = A + B \cdot W + C \cdot W^2 + e_i$$

$$X_{DT,i} = X_{\log(1/R),i} - \hat{X}_{\log(1/R),i}$$

where

$X_{DT,i}$ is the DT spectrum value at wavelength i.

$\hat{X}_{\log(1/R),i}$ is the estimated value of $X_{\log(1/R),i}$ obtained from the quadratic function.

W is the wavelength value.

2.4.3. Combinations ADT and DTA

ADT and DTA are calculated applying both AS and DT sequentially in different order. ADT is calculated directly by the softwares used, while DTA was obtained applying separately both transformations. ADT transformations result in a spectrum that has mean zero and non-unitary variance, while DTA correction gives a spectrum with non-zero mean and variance of one. Both ADT and DTA were found to be related by a scaling factor [32].

2.4.4. Multiplicative scatter correction (MSC) [31]

Standard MSC (SMSC) is performed calculating a least squared regression between each spectrum and the mean spectrum of the whole data set, thus, it is considered a set-dependent transformation. Afterwards, the regression coefficients are used to correct the spectra, as it is stated below:

$$X_{\log(1/R),i} = a_i + b_i \, \overline{X}_{\log(1/R),i} + e_i$$

$$X_{MSC,i} = \frac{\left(X_{\log(1/R),i} - a_i\right)}{b_i}$$

where

$X_{MSC,i}$ is the MSC spectrum value at wavelength i.

$X_{\log(1/R),i}$ is the log(1/R) spectrum value (raw data) at wavelength i.

$\overline{X}_{\log(1/R)}$ is the mean value of all log(1/R) spectra of the data set at wavelength i.

i is the wavelength.

Weighted MSC (WMSC) [33] is defined as a modification of SMSC that uses a correction for the spectral mean and standardizes at each wavelength. In this transformation, to compute the simple linear regression, the absorbances are weighted according to their standard deviation.

Dhanoa et al. [34] found that MSC and SNV were lineally related, while Helland et al. [35] confirmed the existence of connections among other pre-treatments developed for scatter correction.

2.4.5. Spectral derivatives

Spectral differentiation or derivatives are frequently used in NIR spectroscopy. Hopkins [36] cited, as the main methods used to calculate derivatives, the convolution function developed by Savitzky and Golay [37], the point-difference method used in the Grams software (Thermo Galactic, Salem. NH, USA); and the Norris derivative or "segment-gap" method used in Vision and NSAS softwares (FOSS NIRSystems, Silver Spring, MD, USA) and ISI–WINISI softwares.

ISI derivative math treatments are referred to by a four-digit notation (a, b, c, d), where a is the derivative order, b is the derivative gap, c is the smoothing segment and d is the second smoothing segment [33].

2.5. Calibration development and management of outlier samples

Spectral centering and detection of spectral outliers prior to calibration development were omitted, so as to test the ability of pre-treatments in the detection of these anomalies. All calibrations were obtained for the spectral range 1100–2498 nm and none, 2 or 9 maximum number of outliers elimination passes. Limits for outlier detection were 2.5 and 3 for T and H statistics, respectively [33].

Modified Partial Least Squared (MPLS) calibrations were obtained using an automatic routine implemented with the option "teach automatic sequence" included in ISI software [33]. The calibrations were developed using from 0 to a maximum of 9 passes of automatic outliers (T and H) elimination. T outliers are defined as samples with significant differences between their
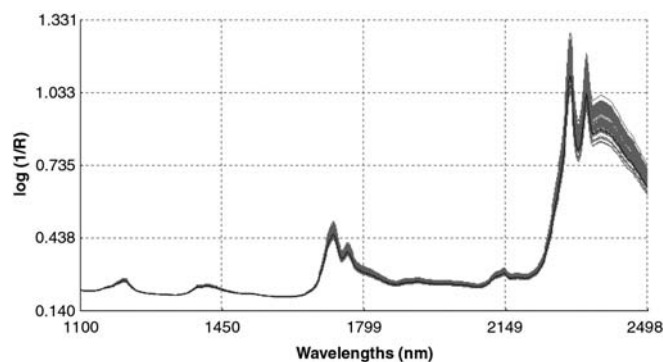

Fig. 1. Reflectance spectra of the reference pig fat thawed and scanned at regular intervals.
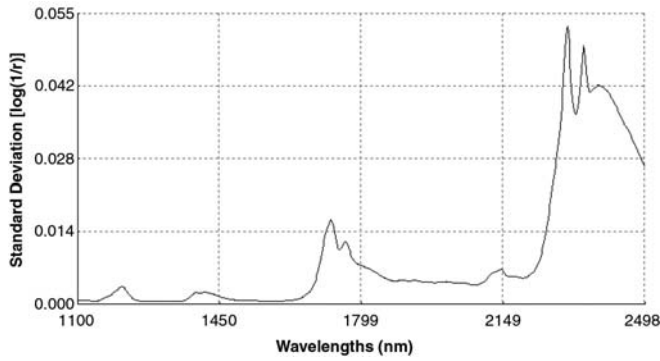
Fig. 2. Standard Deviation of reflectance spectra of the reference pig fat thawed and scanned at regular intervals.

laboratory and predicted values, while H outliers are defined as samples whose spectra show excessive distance ($H>3$) to the spectral centre of the calibration set [33].

2.6. Statistical analysis

Performance of NIRS equations was evaluated by examining the statistical values obtained for calibration and validation with test set procedures. Main calibration statistics are 1-VR (determination coefficient for cross validation) and SECV (standard error of cross validation), while validation was evaluated by SEP (C) (standard error of prediction bias corrected) and R^2 (determination coefficient for validation).

The SEP(C) values were compared according to the procedure published by Fearn [26], based on pairwise comparisons of the standard deviation of the residual vectors coming from two models and on the R^2 between these two vectors. First, the correlation coefficient was found between the two sets of prediction errors (r) and then K was calculated:

$$K = \frac{2(1-r^2)t^2_{n-2,0.025}}{n-2}$$

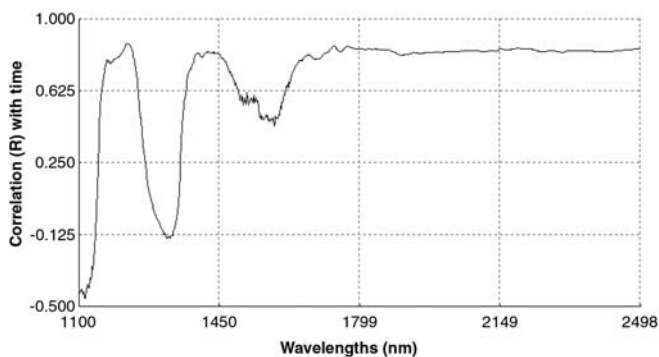where $t^2_{n-2,0.025}$ is the upper 2.5% point of a t distribution for $n-2$ degrees of freedom.

Fig. 3. Correlation coefficients (R) between absorbance values corresponding to sub-samples of the reference pig fat, thawed and scanned at regular intervals along the spectral collection period, and elapsed time since first scan.

Then, L was found

$$L = \sqrt{K + \sqrt{(K^2-1)}}$$

The next two functions define the lower and the upper limits of a 95% confidence interval for the ratio of the true standard deviations. If the interval includes 1, errors are not significantly different at the 5% level:

$$\frac{SEP(C)_1}{SEP(C)_2} \times \frac{1}{L} \quad \text{and} \quad \frac{SEP(C)_1}{SEP(C)_2} \times L.$$

The previous test was compared with the procedure used by Roggo et al. [23]. This method is based on a Fisher test and
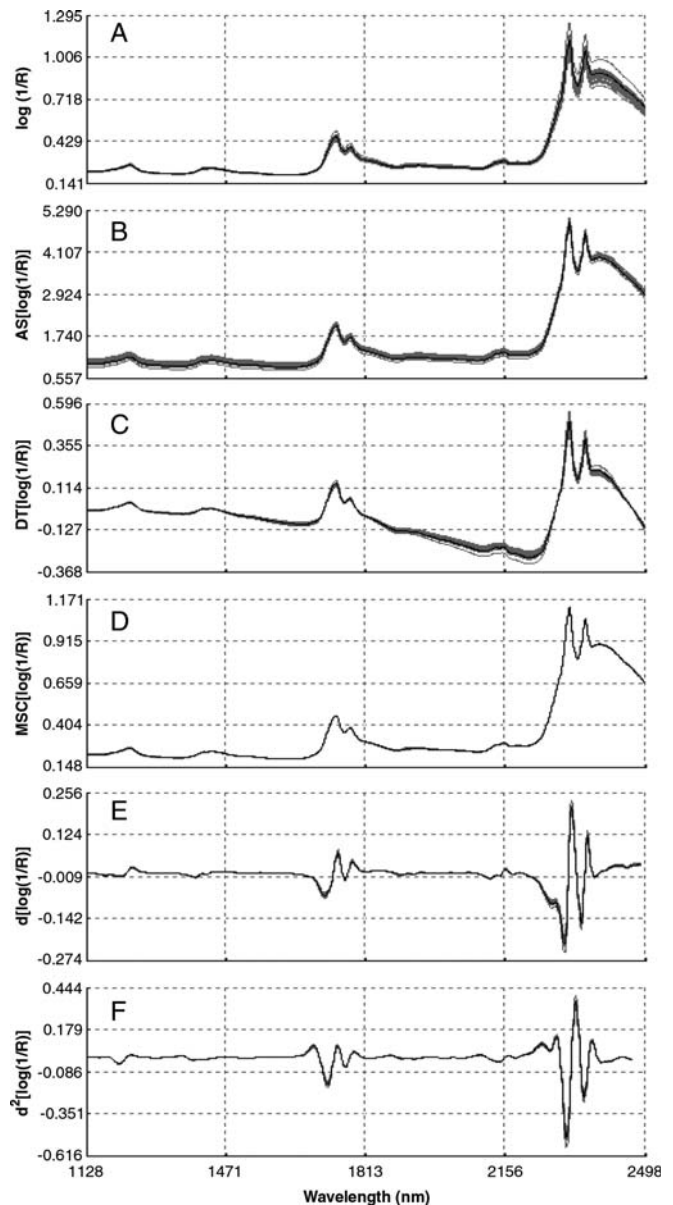

Fig. 4. Reflectance spectra of the Iberian pig fat calibration set. A=raw spectra; B=AS transformed spectra; C=DT transformed spectra; D=standard MSC; E=derivative 1,5,5,1, and F=derivative 2,10,10,1.

Table 3
Maximum and minimum SECV values for C16:0, C18:0, C18:1 and C18:2 calibrations with 0, 2 or 9 maximum number of outliers elimination passes for all pre-treatments; and Standard Error of Laboratory (SEL) for the reference analyses

	C16:0						C18:0					
Elim. passes	0		2		9		0		2		9	
Calibration	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa
Minimum	0.38	5	0.27	17	0.18	38	0.32	5	0.25	15	0.22	7
Maximum	0.45	23	0.31	7	0.30	1	0.36	8	0.32	47	0.30	29
SEL	0.26						0.22					
	C18:1						C18:2					
Elim. passes	0		2		9		0		2		9	
Calibration	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa	SECV	Eqa
Minimum	0.42	19	0.27	44	0.22	3	0.22	8	0.15	15	0.15	14
Maximum	0.45	1	0.32	1	0.28	1	0.28	7	0.20	1	0.20	7
SEL	0.25						0.15					

C16:0: palmitic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid.
SECV: standard error of cross validation.
SEL: standard error of the reference method (gas chromatography).
Eqa: equation number.

defines a confidence interval for errors with non-significant differences with minimum error obtained (Error$_{min}$):

$$\left(\text{Error}_{min}, \text{Error}_{min}\sqrt{F_{1-\alpha,n-1,n-1}}\right)$$

where α is the significance level (5% in this study) and $(n-1)$ the degrees of freedom.

3. Results and discussion

As stated before, there is evidence of the existence of unexpected spectral variation during spectra acquisition. Therefore a preliminary stage explored the spectra plot of the data set corresponding to the reference pig fat sample scanned repeatedly (Fig. 1), where spectral scatter effects were confirmed. In this figure, it could be noticed that spectral variations were higher for wavelengths with higher absorbance values. This fact was confirmed studying the standard deviations of the absorbance data for this set (Fig. 2), where it was observed that the shape of the spectral discrepancies was similar to the average log(1/R) value, indicating that some of the pre-treatments used for the scatter correction could help in their reduction.

Moreover, absorbance values associated with the reference sample showed an increasing trend during the period of analysis, as older spectra generally had lower log(1/R) values. Correlation coefficients of absorbance values corresponding to sub-samples of the reference fat,

Table 4
Mean, minimum and maximum number of samples deleted during calibration development for C16:0, C18:0, C18:1 and C18:2 with 2 or 9 maximum number of outliers' elimination passes

		C16:0		C18:0		C18:1		C18:2	
	Passes	2	9	2	9	2	9	2	9
Del. samples	Mean	15	31	11	20	14	24	10	13
	Minimum	11	12	6	10	11	16	8	8
	Maximum	19	42	15	30	18	39	13	25

C16:0: palmitic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid.

scanned at regular intervals along the spectral collection period, with elapsed time since first scan proved to be medium-high for all wavelengths at the second half of the spectral range (Fig. 3), showing an important effect of time on the incidence of spectral variations. Negative correlation coefficients found at the beginning of the spectra could be a consequence of the presence of a spectral curvature effect (illustrated by Fig. 4D) together with the distortions caused by reduced absorbance values at the mentioned region. The observed drift in absorbance values could be caused by the loss of reflectance properties of the aluminium surface placed at the back of the analysis cell used or even by slight changes in the optical features of the instrument.

The effect of data pre-treatments was easily seen by visual inspection of transformed spectra, as shown in Fig. 4. The degree of spread observed in raw data (log 1/R) for the pig fat spectra set along the Y axis was reduced by the use of any of the pre-treatments studied. Nevertheless, the greatest reduction in the spectral dispersion was achieved with the application of MSC and derivatives. DT and MSC reduced spectral variation maintaining peaks position in relation to the log 1/R raw data. DT transformation, when applied to scattering products like intact or milled animal feeds, usually corrects the observed curvature of their spectral baselines [30], but in this case, where original spectra showed an almost flat baseline, DT caused a decreasing baseline effect. Application of any of both derivatives changed drastically spectral shapes, duplicating original peaks into positive and negative going features and introducing new peaks.

However, reduction of the observed spread in a spectral data set for a certain pre-treatment is not a warranty for a good result in the calibration development procedure. It is impossible to know beforehand which pre-treatment will lead to the most accurate model with a certain calibration set. Thus, we used the option "teach automatic sequence" included in ISI software to create a macro command which allowed the calculation of 49 regression models for each analytical parameter with just one order. This option has an invaluable utility, as for this paper it was necessary to develop and validate 588 equations with more than 300 calibration samples. Although computers are able to perform calculations in a reasonable period of time, each outlier elimination pass implies a new calibration computation and, consequently, an increment in the final computation time.

Table 5
SEP(C) maximum, minimum (lower confidence limit) and upper confidence limit for C16:0, C18:0, C18:1 and C18:2 calibrations with 0, 2 or 9 maximum number of outliers elimination passes

	C16:0						C18:0					
Elim. passes	0		2		9		0		2		9	
Calibration	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa
Minimum	0.35	18	0.39	13	0.37	19	0.27	36	0.26	5	0.26	1
Maximum	0.43	1	0.46	4	0.45	2	0.34	20	0.34	8	0.34	8
Upper CL	0.48		0.53		0.50		0.37		0.35		0.35	
	C18:1						C18:2					
Elim. passes	0		2		9		0		2		9	
Calibration	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa	SEP(C)	Eqa
Minimum	0.57	5	0.58	20	0.57	27	0.27	47	0.23	35	0.27	47
Maximum	0.65	22	0.67	47	0.68	47	0.36	2	0.43 *	2	0.43 *	2
Upper CL	0.78		0.79		0.78		0.37		0.31		0.37	

C16:0: palmitic acid; C18:0: stearic acid; C18:1: oleic acid; C18:2: linoleic acid.
SEP(C): standard error of prediction bias corrected.
CL: confidence limit.
Eqa: equation.
 * Indicates significant differences with minimum error at $P = 0.05$.

Although we are aware of the importance of a detailed discussion about the causes and management rules for outliers detected during calibration development for products like fats, that can be affected by huge number of error sources [20,21], in the present paper this phase was omitted due to the great number of calibrations to be tested. Nevertheless, using an automatic deletion procedure, it was possible to obtain regression models with 0, 2 or 9 maximum number of outlier elimination passes. This fact allowed us to test the effect of spectral pre-treatments on the number of samples detected as *T* or *H* outliers.

The incidence of the combination of spectral pre-treatments used during calibration can be analysed with the use of Table 3. There were found differences between maximum and minimum cross validation errors (SECV) for all parameters and number of outlier elimination passes, as a result of the effect of the combination of the mathematical pre-treatments applied. Elimination of outliers, generally, improved the calibration statistics, allowing SECV to reach values close to the calculated standard error of laboratory (SEL) for the reference method (gas chromatography). It was observed that results obtained with 2 or 9 maximum number of outliers elimination passes were reasonably similar, except for palmitic acid calibrations, which could be an indication of the elimination of main outlier samples during the first two deletion passes.

In relation to the ability of the different pre-treatments to detect real outliers, Table 4 shows the mean, minimum and maximum number of samples deleted during calibration development for 2 or 9 maximum number of automatic outlier elimination passes. The selection of 9 passes as maximum for the elimination of outliers was done for comparison purposes only, and it is not recommended. However this option does not suppose the mandatory reaching of this limit in the development of calibrations, being generally selected a lower number of passes. The average number of deleted samples with a maximum of two elimination passes for all calibrations varied between 10 and 15, which represents a 3–5% of the initial calibration set. Important differences were not detected between the maximum and minimum number of samples removed, so it could be presumed that they were real anomalous samples. With a maximum of 9 elimination passes, there were removed an average number of outliers that varied between 13 and 31, which stands for 4–10% of the calibration set. One could

thus think that some pre-treatments show higher abilities to detect real outliers during calibration development, while other transformations lead to the detection of false anomalies, like samples with extreme laboratory values. The importance of the use of an appropriate mathematical transformation is emphasised, as elimination of false outliers during calibration could cause a reduction in the prediction ability of calibrations [38]. Nevertheless, differences in the number of deleted samples with 2 or 9 maximum number of elimination passes were slight, thus we could have an estimation of the magnitude of the real number of anomalies present in the calibration data set.

Influence of pre-treatments on the predictive ability of calibrations was determined with paired comparisons of the minimum SEP(C) value and the remaining SEP(C) values, using the statistical tests proposed by Fearn [26] and Roggo et al. [23]. As conclusions obtained with both statistical tests were coincident, all information was summarised in Table 5, including maximum and minimum SEP(C) values for both analytical parameters and the different maximum number of outlier elimination passes, and also the SEP(C) upper confidence limit value, calculated as $SEP(c)_{min} x \sqrt{F_{0.95,29,29}}$. According to Roggo et al. [23] all models which have SEP(C) between SEP $(C)_{min}$ and the upper confidence limit were not significantly different. Table 5 shows that the prediction errors were also affected by the pre-treatment used, but differences between maximum and minimum were marginal for all parameters except for linoleic acid.

Comparison of prediction errors for palmitic acid showed that there were not significant differences for any number of outlier elimination passes. Minimum errors were obtained with calibrations developed with derivative 2,10,5,1 combined with pre-treatments AS, ADT and SMSC, for no outlier elimination passes; derivative 2,10,10,1 alone or combined with AS, ADT, DTA and SMSC, for 2 maximum number of outlier elimination passes; and derivative 2,10,10,1 combined with SMSC and AS, for 9 maximum number of outlier elimination passes. It is clearly shown that best results were obtained with the use of a second derivative: 2,10,5,1 for no outliers' deletion, and 2,10,10,1 for any number of outliers elimination passes. On the contrary, highest errors were obtained for AS, ADT, first derivatives and other pre-treatments applied alone. Other validation statistics like bias, slope and determination coefficient fulfilled the control limits suggested by Shenk

et al. [39] only for the calibrations developed without outlier elimination passes. This fact could be explained as the control limits applied for SEP (C) seem to be more restrictive for models with better calibration statistics (lower calibration errors).

SEP(C) values obtained for stearic acid calibration were not significantly different. Best results were observed with the application of pre-treatments DTA+2,10,5,1; DTA; and none, DTA, SMSC, 2,5,5,1 and DT+2,5,5,1; for 0, 2 and 9 maximum number of outlier elimination passes, respectively. A clear pattern was not observed associated with the highest prediction errors, even though the use of derivative 1,5,5,1 or AS produced higher errors in many cases. In relation to the control limits for validation statistics, a 100% conformity was observed without elimination of outliers, whereas with a maximum of 2 elimination passes, only one situation arose where SEP(C) resulted in values higher than the calculated limit (1.3 times SECV) [39]. The use of more elimination passes incremented the number of non conformities in relation to SEP(C) up to 20.

Significant differences were not found for the validation errors of oleic acid calibrations at any level of outlier elimination passes. Minimum errors for each outlier elimination level were obtained with calibrations developed with DTA and SMSC; DT combined with derivative 1,5,5,1; and DT combined with derivative 2,10,10,1; for 0, 2 and 9 maximum number of outlier elimination passes, respectively. Prediction errors for 2 or 9 maximum number of outliers elimination passes were superior for the combinations ADT+2,10,5,1 and WMSC+2,5,5,1, giving quite poor prediction performance when using the second derivative 2,5,5,1. Furthermore, all SEP(C) values were higher than the calculated limit for all calibrations tested.

Probably, the most interesting results appeared with calibrations developed for linoleic acid. Firstly, it was the only parameter that presented significant differences for the prediction errors with any number of outlier elimination passes. The combination of pre-treatments that led to lower validation errors for each outlier elimination level were WMSC combined with derivative 2,5,5,1; DTA combined with derivative 1,10,10,1; and WMSC combined with derivative 2,5,5,1; for 0, 2 and 9 maximum number of outlier elimination passes, respectively. Moreover, all spectral transformations that included derivatives 2,5,5,1 or 1,5,5,1, combined or alone, produced lower SEP(C) values, showing the influence of the derivative parameters (order, gap and smoothing segment) on the performance of calibrations. On the contrary, the application of scatter corrections like AS, DTA, SMSC, WMSC, ADT and DT alone or the use of raw spectra resulted in higher validation errors. In relation to the compliance with validation control limits, there were detected only discrepancies for SEP(C) values. In this sense, only a few combinations of pre-treatments that included derivatives 1,5,5,1 or 2,5,5,1 (except DTA+2,5,5,1) satisfied error limit criteria.

It was possible to find a pattern for the combination of pre-treatments (especially derivatives) that provided the best and worst results in validation tests, as it has been concluded for other agro-food products [23,24]. Moreover, the use of certain derivatives alone or in combination with other pre-treatments showed an important effect on validation results for some analytical parameters.

4. Conclusions

Despite that performance of prediction models showed variations with the mathematical pre-treatment used, general significant differences were not detected with the tests applied, except for linoleic acid.

The effect of spectral pre-treatments on the predictive ability of NIRS calibration models should be measured not only by

their ability to improve the detection of real outliers during calibration but also by the degree of adjustment among the constituent values and the transformed spectral data.

The best pre-treatments in calibration and in prediction usually differ. Therefore, when making evaluation of pre-treatments it would be desirable to use some automatic software routines that permit an efficient evaluation of different validation sets and/or the execution of a large number of cross validation passes.

As NIR spectra of pig fats is characterized by the presence of sharp peaks susceptible to changes caused by unexpected variations, the optimisation of the derivatives used (order, gap and smoothing segment) is essential for the implementation of robust calibrations.

Unexpected spectral variations are partially corrected with the use of pre-treatments in calibration, but not as well as using other chemometric strategies, like repeatability files.

References

[1] A. Fernández, E. De Pedro, N. Núñez, L. Silió, J. García-Casco, C. Rodríguez, Meat Sci. 64 (2003) 405–410.
[2] I. González-Martín, C. González-Pérez, J. Hernández-Méndez, N. Alvarez García, Meat Sci. 65 (2003) 713–719.
[3] I. González-Martín, C. González-Pérez, N. Alvarez-García, J.M. González-Cabrera, Meat Sci. 69 (2005) 243–248.
[4] R. Cava, J. Ruíz, C. López-Bote, L. Martín, C. García, J. Ventanas, T. Antequera, Meat Sci. 49 (suppl. 1) (1997) S87–S99.
[5] I. Díaz, J.A. García Regueiro, M. Casillas, E. De Pedro, Food Chem. 55 (1996) 383–387.
[6] C. García, J.L. Berdague, T. Antequera, C. López-Bote, J.J. Córdoba, J. Ventanas, Food Chem. 41 (1991) 43–48.
[7] E. De Pedro, A. Garrido, I. Bares, M. Casillas, I. Murray, in: K.I. Hildrum, T. Isaksson, T. Naes, A. Tandberg (Eds.), Proceedings of the 5th International Conference on Near infra-red Spectroscopy. Bridging the Gap Between Data Analysis and NIR Applications, Haugesund, Ellis Horwood, Chichwster, UK, 1992, pp. 345–348.
[8] C. Hervás, A. Garrido, B. Lucena, N. García, E. De Pedro, J. Near Infrared Spectrosc. 2 (1994) 177–184.
[9] I. González-Martín, C. González-Pérez, J. Hernández-Méndez, N. Alvarez-García, J.L. Hernández-Andaluz, Anal. Chim. Acta 453 (2002) 281–288.
[10] L.P. Houmøller, D. Kristensen, H. Rosager, Talanta (2006), doi:10.1016/j.talanta.2006.05.066.
[11] A. Jiménez Márquez, A. Molina Díaz, M.I. Pacual Reguera, Sens. Actuators B 107 (2005) 64–68.
[12] D. Cozzolino, I. Murray, A. Chree, J.R. Scaife, LTW 38 (2005) 821–828.
[13] M. Pla, P. Hernández, B. Ariño, J.A. Ramírez, I. Díaz, Food Chem. 100 (2007) 165–170.
[14] C.E. Realini, S.K. Ducket, W.R. Windham, Meat Sci. 68 (2004) 35–43.
[15] A. Dalle Zotte, P. Berzaghi, L.M. Jansson, I. Andrighetto, Anim. Feed Sci. Technol. 128 (2006) 108–121.
[16] C.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman, Chemometrics: A Textbook, Elsevier, Amsterdam, The Netherlands, 1988.
[17] P. Geladi, E. Dåbakk, J. Near Infrared Spectrosc. 3 (1995) 119–132.
[18] P.C. Williams, K.H. Norris, Near-Infrared Technology in the Agricultural and Food Industries, American Association of Cereal Chemists, St. Paul, Minnesota, USA, 2001, pp. 171–185.
[19] J.S. Shenk, J. Workman, M. Westerhaus, Handbook of Near-Infrared Analysis, Marcel Dekker, New York, USA, 1992, pp. 383–431.

[20] A. Garrido-Varo, J. García-Olmo, M.D. Pérez-Marín, Near Infrared Spectroscopy in Agriculture, Monograph, ASA-CSSA-SSSA, Madison, WI, USA, 2004, pp. 487–558.

[21] M.D. Pérez Marín, E. De Pedro, J. García-Olmo, A. Garrido, in: A.M.C. Davies, R.K. Cho (Eds.), Proceedings of the 10th NIR International Conference, Near Infrared Spectroscopy: The Future Waves, NIR Publications, Chichester, UK, 2002, pp. 473–475.

[22] P. Dardenne, G. Sinnaeve, V. Baeten, J. Near Infrared Spectrosc. 8 (2000) 229–237.

[23] Y. Roggo, L. Duponchel, B. Noe, J.P. Huvenne, J. Near Infrared Spectrosc. 10 (2002) 137–150.

[24] Y. Roggo, L. Duponchel, C. Ruckebuch, J.P. Huvenne, J. Mol. Struct. 654 (2003) 253–262.

[25] S.R. Delwiche, J.B. Reeves, J. Near Infrared Spectrosc. 12 (2004) 177–182.

[26] T. Fearn, NIR News 7 (5) (1996) 5–6.

[27] J. García-Olmo, A. Garrido, E. De Pedro, J. Near Infrared Spectrosc. 9 (2001) 49–62.

[28] J.S. Shenk, M.O. Westerhaus, Crop. Sci. 31 (1991) 1694–1696.

[29] H. Martens, T. Naes, Multivariate Calibration, John Wiley and Sons, Chichester, UK, 1989.

[30] R.J. Barnes, M.S. Dhanoa, S.J. Lister, Appl. Spectrosc. 43 (1989) 772–777.

[31] H. Martens, S.A. Jensen, P. Geladí, Proceedings of the Nordic Symposium on Applied Statistics, Stokkand Forlag Publishers, Stavanger, Norway, 1983, pp. 205–233.

[32] M.S. Dhanoa, S.J. Lister, R.J. Barnes, Appl. Spectrosc. 49 (1995) 765–772.

[33] J.S. Shenk, M.O. Westerhaus, Routine Operation, Calibration, Development and Network System Management Manual, NIRSystems, Inc., Silver Spring, MD, USA, 1995.

[34] M.S. Dhanoa, S.J. Lister, R. Sanderson, R.J. Barnes, J. Near Infrared Spectrosc. 2 (1994) 43–47.

[35] I.S. Helland, T. Naes, T. Isaksson, Chemometr. Intell. Lab. Syst. 29 (1995) 233–241.

[36] D.W. Hopkins, Near Infrared Anal. 2 (1) (2001) 1–13.

[37] A. Savitzky, M.J.E. Golay, Anal. Chem. 36 (1964) 1627–1638.

[38] J.S. Shenk, M.O. Westerhaus, Analysis of Agricultural and Food Products by Near Infrared Reflectance Spectroscopy. Monograph, NIRSystems Inc., Silver Spring, MD, USA, 1995.

[39] J.S. Shenk, M.O. Westerhaus, S.M. Abrams, in: G.C. Marten, J.S. Shenk, F.E. Barton (Eds.), Near Infrared Spectroscopy analysis of forage quality, ARS Agriculture Handbook n° 643, USDA, Washington DC, USA, 1989, pp. 104–110.