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Carbon stable isotopes, fatty acids and the use of NIRS to differentiate IBERIAN pigs

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ABSTRACT

This study explores the viability of the application of Near Infrared Spectrometry (NIR) for the rapid prediction of the ratio of $^{13}C/^{12}C$ stable isotopes and fatty acid composition in Iberian pigs. The potential use of this technique for distinguishing samples according to the duration of the *montanera* period was also studied. Subcutaneous fat samples from 50% and 100% Iberian pigs allowed to feed freely during different *montanera* periods were analyzed: 24 biopsies were taken prior to the *montanera* and 106 samples were taken after this feeding period. The results show significant correlations between $\delta^{13}C$ (‰) and several fatty acids. Furthermore, it is possible to differentiate samples taken from pigs reared using different feeding regimes by analyzing the data obtained from the NIR spectra or by applying an Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) on data on $\delta^{13}C$ (‰) and fatty acids in subcutaneous fat.

1. Introduction

The Iberian pig is of great importance to the Spanish economy, and has traditionally been consumed as sausages and, in particular, as Iberian acorn-fed ham, which is the product that is the most well-known. The quality label of 'Iberian pig' includes the pure Iberian breed and Iberian crossbreeds, with Duroc being the most commonly used breed for creating hybrids. The most important characteristic of the Iberian pig is that it produces a high amount of intramuscular fat which gives its meat a veined appearance and its distinctive juiciness and flavor. This quality is due not only to the breed, but to also to a particular rearing method known as *montanera*, where Iberian pigs during the last stage of their lives are allowed to roam freely and feed on the natural resources (mainly holm oak and cork acorns and grass) available in the *dehesa* ecosystem. The feeding conditions influence the endogenous and exogenous synthesis of fatty acids in the pigs (Cava et al., 1997; Ruiz et al., 1998), and it is known that the longer the *montanera* period, the higher the C18:1n-9, C18:3n-3 and ΣMUFA content and the lower C18:2n-6, n-6 and n-6/n-3 content present in the animals (Daza, Ruiz-Carrascal, Olivares, Menoyo, & López-Bote, 2007; Rey, Daza, López-Carrasco, & López-Bote, 2006). It is for this reason that the fatty acid composition of Iberian pig subcutaneous fat has been used as a suitable tool for classifying animals in accordance with the feed used to fatten them (Delgado-Chavero, Zapata-Márquez, García-Casco, & Paredes-Torronteras, 2013). However, Spanish regulations governing Iberian products do not include the use of fatty acid profiles for distinguishing commercial categories (Official State Bulletin [BOE], 2014); although current legislation establishes how the purity of the pig breeds is controlled as well as extensive production systems linked to a *dehesa* environment by means of inspections and certifications issued by the National Accreditation Entity (ENAC).

Carbon isotope ratios can also provide information about an animal's diet (De Niro & Epstein, 1978), with the C4 and C3 plant material content in feed having a great influence on the animals' $\delta^{13}C$ (‰) value (Bahar et al., 2005; Bahar et al., 2009). For this reason, studying the carbon ($\delta^{13}C$) and sulphur ($\delta^{34}S$) isotopic ratio has also been used as a method to distinguish animals according to their feeding regime (González-Martín, González-Pérez, Hernández Méndez, Marqués-Macias, & Sanz Poveda, 1999; González-Martín, González, Hernández Méndez, Recio Hernández, & Sabio Rey, 1997; González-Martín, González Pérez, Hernández-Méndez, & Sánchez González, 2001; López-Bascón et al., 2015). The analysis of fatty acids and isotopes of fatty acids has also been studied to distinguish Iberian pigs according to the type of feed that is used during the fattening process (Delgado-Chavero et al.,

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2013; Recio, Martín, & Raposo, 2013).

Nonetheless, all of these methods present drawbacks owing to the availability of commercially sold fattening concentrates, which mimic the composition of the main fatty acids (palmitic, stearic, oleic and linoleic) of acorn and grass and can distort analytical results (Delgado-Chavero et al., 2013). Moreover, differences can arise in the fatty acid profile between years due to the variability of the quality of acorns used to feed the pigs.

Several other analytes have been assessed for their utility in determining the feed consumed by pigs during the fattening period such as tocopherols (Rey et al., 2006), volatile compounds (Sánchez del Pulgar et al., 2013) and the triacylglycerol profile (Bayés-García et al., 2016; Viera Alcaide, Vicario, Escudero Gilete, Graciani Constante, & León Camacho, 2008). However, the further processing industry continues to analyze and make use of pork fatty acid profile as it greatly influences both fresh and dry cured color and fat oxidation (Gilles, 2009; Melgar, Cid, Astiasarán, & Bello, 1991; Ruiz-Carrascal, Ventanas, Cava, Andrés, & García, C., 2000; Ventanas, Andrés, Cava, Tejeda, & Ruiz, 1999; Ventanas, Ventanas, Jurado, & Estévez, 2006). Moreover, determination of the fatty acid profile also helps processors decide on salting and drying times during the production of dry-cured ham (Cava López & Andrés Nieto, 2001).

In addition to the above mentioned methods, Near infrared spectroscopy (NIR) may be considered an interesting alternative as it is much faster and cheaper to use. Additionally, this tool has been widely used to assess the characteristics of meat and the quality of meat products (Prieto, Roehe, Lavín, Batten, & Andrés, 2009). In the case of Iberian pigs, this technique has been used to quantify protein, fat and moisture contents (Zamora-Rojas, Garrido-Varo, De Pedro-Sanz, Guerrero-Ginel, & Pérez-Marín, 2011), fatty acid profiles (De Pedro, Garrido, Bares, Casillas, & Murray, 1992; De Pedro, Garrido, Lobo, Dardenne, & Murray, 1995; Fernández-Cabanás, Garrido-Varo, García Olmo, Pedro, & Dardenne, 2007; Gonzalez-Martin, Gonzalez Perez, Hernandez Mendez, & Alvarez-Garcia, 2003) as well as to determine the pig's diet during the process of fattening (Arce et al., 2009; Hervás, Garrido, Lucena, García, & De Pedro, 1994; Zamora-Rojas et al., 2011; Zamora-Rojas, Pérez-Marín, De Pedro-Sanz, Guerrero-Ginel, & Garrido-Varo, 2012).

Taking all of the above into consideration, the first aim of this work was to study the fatty acid profile and the progression of the carbon isotopic ratio according to the production system used for pig rearing and the days during which the *montanera* took place. For this purpose, tissue samples were taken by means of biopsies prior to the onset of the *montanera* and then later on in the slaughterhouse after the animals had experienced different *montanera* durations. This approach has previously been used by other authors to test the influence of diet on fatty acid composition (Barea, Isabel, Nieto, López-Bote, & Aguilera, 2013; Pascual et al., 2006). However, the effect of different *montanera* feeding times on the fatty acid profile and on the carbon isotopic ratio has not until now been considered. In addition, the feasibility of predicting fatty acid composition and $\delta^{13}C$ (‰) in samples taken before and after the *montanera* was also studied. Prediction of the 13C/12C stable isotope ratio has recently been performed (González-Martín et al., 2021) in subcutaneous fat but not through the use of biopsies. Moreover, to our knowledge, no study has established whether there are significant relationships between $\delta^{13}C$ (‰) and fatty acids and whether these relationships change due to a *montanera* feeding regime.

Finally, the most relevant purpose of this work was to study the feasibility of differentiating samples according to the production systems employed and the duration of the *montanera*, since the more days the animals spend feeding freely on acorns and grass, the higher the quality of the final products. This approach differs from previous work comparing animals fed on acorns and grass with animals fed exclusively on formulated feed (*cebo*) or given formulated feed just before being slaughtered (*recebo*) (Delgado-Chavero et al., 2013; Recio et al., 2013; Ruiz et al., 1998), or studies comparing the results obtained after feeding on different types of acorn vs grass (Rey et al., 2006). To this end, different methodologies, such as using only NIR spectra, only fatty acid composition or fatty acids plus the isotopic ratio, were compared to determine the optimal method for differentiating animals according to the feeding regime employed.

2. Materials and methods

2.1. Samples

Subcutaneous fat samples were taken from Iberian pigs in three different monitored farms during the 2018–2019 season. All animals were *montanera*-fed during the last fattening stage (diet based on acorns, grass and other resources naturally found in a *dehesa* ecosystem) in the same geographic area (Badajoz, Spain). Farm 1 rears pigs that are 100% Iberian and carries out a *montanera* over a course of 68 days, farm 2 also rears pigs that are 100% Iberian but the *montanera* period is 84 days and farm 3 rears 50% Iberian-Duroc crossbreeds and the *montanera* is for 120 days. All batches were identified by means of the ITACA System and ear tags, which are assigned according to feeding batches. This requirement is mandatory and regulated within the Iberian pig Quality Standard (Order AAA/1549/2014, of 28 August) for all pig farmers. This identification number allows National Accreditation Entity ENAC to monitor and certify each batch and to ensure its traceability and that the requirements of care in production and quality regulated by the Iberian pig Quality Standard are met. The numbers of the different batches were: Farm 1 - LOTE-EXP-170425-245,768; Farm 2 - LOTE-EXP-170913-274,668; and Farm 3 - LOTE-EXP-171026-285,571.

The samples were collected at two different time points, with the first sample collection involving biopsies carried out on live animals (24 animals from each farm). The biopsies were taken the first day of *montanera* and the pigs had a live weight ranging from 92 to 115 kg. All the samples were taken on the farm by a veterinarian from the coccyx area following the method described by Bosch-Puig, Puigvert-Colomer, Tor-Naudí, Villalba-Mata, and Estany-Illa (2008). Briefly, the biopsy site was cleaned, shaved and disinfected (chlorhexidine digluconate). The biopsy was taken using the spring loaded biopsy equipment (PPB-U Biotech, Nitra, Slovakia). Once the sample was extracted, the biopsy site was again disinfected. The samples were collected from the cannula and introduced in individual plastic tubes with the animal reference number, cleaning properly the cannula before taking the next sample.

The second sampling was of subcutaneous fat taken in the coccyx area of the pig carcasses, a few centimeters from the tail following the line of the vertebral column. Just before being slaughtered, the animals had a live weight ranging from 130 to 160 kg. A total of 72 biopsied samples were collected (24 animals from each of the three farms) and 106 samples were collected from the subcutaneous fat of the carcasses (30 samples from farm 1, 31 samples from farm 2 and 45 samples from farm 3).

The samples were kept frozen until analysis (−28 °C). Since the tissue size of the biopsies was small, 3 biopsies from different animals from the same farm were pooled for analysis. After the removal of impurities from the skin and loin, the fat samples collected after slaughter were homogenized using a Polytron blender until a uniform mixture was obtained. The mixtures were then melted in a microwave oven according to the method described by González-Martín, González-Pérez, Hernández-Méndez, Álvarez-García, and Merino Lázaro (2002).

2.2. Instrumentation and methods

2.2.1. Fatty acid analysis

To quantify the fatty acids, 0.1 g of extracted fat were weighed. The fatty acids (FA) were methylated using the method described by Murrieta, Hess, and Rule (2003). Quantification was performed by gas chromatography (gas chromatograph (GC) 6890 N; Agilent Technologies, Santa Clara, CA), using a 100 m × 0.25 mm × 0.20 μm capillary column (SP-2560; Supelco, Bellefonte, PA). The chromatographic

conditions were as described in a previous study (González-Martín, Vicente Palacios, Revilla, Vivar-Quintana, & Miguel Hernández-Hierro, 2017). Using this method, the fatty acids ranging from C12:0 and C22:6 were determined. They were identified by comparing the retention times with the corresponding standards (Sigma Aldrich, Steinheim, Germany). The results were expressed as a percentage, according to the peak areas of the chromatogram.

2.2.2. Stable isotope analysis

The HCOS EURO EA 3000 elemental analyzer with a high temperature combustion system and a dilution system connected to a continuous gas flow isotope mass spectrometer (ISOPRIME, Micromass) was used to analyze stable carbon isotopes ($\delta^{13}C$). The natural abundance of ^{13}C was expressed as $\delta 13C = [(13R \text{ sample} / 13R \text{ standard}) - 1] 1000$, where $^{13}R = {}^{13}C / {}^{12}C$ according to the international PDB standard with a precision (2σ) of 0.05–0.08‰ from CO_2 measurement in a double-beam spectrometer.

2.2.3. Near infrared spectrometry spectra (NIRS) register

NIRS were determined using a Foss NIR System 5000 and the fat extracted from the biopsied tissue and from the subcutaneous fat samples. The fat was thermostatted in a bath at 30 °C and 15 μl of liquid fat were collected. Circular capsules with an optical path length of 0.1 nm (cam-lock cells) were used to introduce each sample into the analyzer. Spectra were recorded in triplicate in reflectance mode between 1100 and 2498 nm at 2 nm intervals with 32 scans. All spectra were presented as the logarithm of the reciprocal reflectance (log 1/R) (R = reflectance). The Win ISI 1.50 software package was used for spectra collection, data manipulation and chemometric analysis. For the NIR calibrations, the samples were randomly divided into two groups: the calibration group comprising 80% of the samples (104 samples) and the validation group with the remaining 20% (26 samples).

2.3. Data analysis. Chemometric methods

Statistically significant differences in fatty acid content and δ13C (‰) values, due to breed 100% Iberian ($n = 61$) vs. 50% Iberian ($n = 45$), the *montanera* before ($n = 24$) or after ($n = 106$) and the feeding regime farm 1 ($n = 30$), farm 2 ($n = 31$), farm 3 (n = 45) and their interactions, were determined by the General Linear Model (GLM) using the SPSS 25 package (IBM, Chicago, IL, USA).

2.3.1. Discriminant analysis

Two discriminant analysis models were used. First, information from the NIR spectra was used and spectral differentiation was obtained using the RMS X (the square root of the residual mean squared (RMS) was calculated to obtain the RMSX residuals). This measure is useful for detecting spectral variation that is not similar to the spectral variation in the good product data set (Nørgaard, Haunstrup, Petersen, Weimann, & Sørensen, 2014). In this case WinISI 4.0 software was used.

Secondly, SIMCA-P software version 14.1 (Umetrics) was used to carry out the chemometric analysis of $\delta^{13}C$ (‰) and fatty acid values of each fat sample extracted from the biopsied tissue and from the subcutaneous fat samples. A Principal Component Analysis (PCA) was first applied to each monitored category (farm 1, farm 2 and farm 3) to allow the identification of outliers (Alonso et al., 2008). Once outliers were identified and removed by PCA analysis, an Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed on the entire dataset (106 subcutaneous fat samples or 130 biopsied tissue and subcutaneous fat samples). The data were randomly divided into a training set (80% of the samples) to build the discriminant model and a validation set (20% of the samples selected to include a balanced number of samples from the three groups) to test the model performance. The best OPLS-DA model was chosen considering the discrimination ability given by the number of correctly classified samples and the Q2 parameter. The quality assessment statistic (Q2) is the result of the cross-validation and provides a qualitative measure of the consistency between the predicted and original data (Worley & Powers, 2013).

2.3.2. NIR analysis

Calibration models were obtained using the NIR spectra and chemical data (13C isotopes and fatty acids) for each sample. Sample spectra were processed by PCA to detect outliers. The global Mahalanobis (H) distance considered spectra with an $H \geq 3.0$ standard units from the mean spectrum as outliers (Shenk & Westerhaus, 1996). The use of this standardized H statistic entailed a nearly inexistent risk of including samples that did not belong to the population in the set from which the calibration equations would be obtained. In addition, samples that had a predicted residual value of $T \geq 2.5$ in the chemical data were considered different from the population and removed from the set (Williams & Norris, 1987). Once the samples were removed according to the H-criterion for the spectra and the T-criterion for the chemical data, the statistical parameters of the calibration were obtained. The modified partial least squares (MPLS) regression method was used for the calibration of fatty acids and $\delta^{13}C$ (‰) (Martens & Naes, 1989). The whole region of the spectrum was used; several corrections based on mathematical treatments and scatter corrections (standard normal variant, SNV; De-trending, DT; multiplicative spread corrections, MSC; first derivative and second derivative) were applied. To avoid over-fitting in the development of the MPLS equations, a cross-validation was performed to make 7 groups of samples. The selection criterion to obtain the best equations for each of the components was the highest multiple correlation coefficient (RSQ) and the lowest calibration and cross-validation standard error (SEC and SECV). The equations obtained were applied to the validation set to obtain a corrected standard error of prediction (SEPC) and a bias (the residual mean defined as the distance between the laboratory value and the value predicted by the equation) for each component. The method robustness was checked by applying the calibration equations obtained during the study to the NIR spectra of new samples that did not belong to the calibration group. The predicted values were then compared with the reference data. The method for analyzing NIRS and the reference data were compared using Student's *t*-test for paired values. The *P*-value, residual mean and root mean squared error (RMSE) values were calculated.

3. Results and discussion

3.1. Stable carbon isotopes

Terrestrial plants assimilate atmospheric CO_2 in two different ways: by means of C_s cycle (Calvin-Benson) or by means of C_4 cycle (Hatch-Slack) which is energetically more efficient.

C3 plant tissues are depleted in CO_2 in relation to the atmosphere to about 19%, while the C4 plant tissues are depleted to about 4%. Indeed, plants preferentially assimilate the lighter and more abundant isotope ^{12}C. The $\delta^{13}C$ value for atmospheric CO_2 is 0‰ (although in laboratories the PDB standard is used as a reference, which refers to the Cretaceous belemnite formation at Pee Dee, South Carolina, USA). This means that the $\delta^{13}C$ ‰ values are negative when compared to that of atmospheric CO_2. As a consequence, the different diets of humans or animals in different geographical regions are reflected in the isotopic depletion in their tissues. This phenomenon is called isotopic fractionation.

Table 1 shows the results of the $^{13}C / {}^{12}C$ stable isotope ratio in fat extracted from the biopsied samples (before the *montanera*) and subcutaneous fat samples (after the *montanera*), expressed as $\delta^{13}C$ (‰). These results correspond to the three stockbreeding farms rearing pigs that were either 100% Iberian (farms 1 and 2) or 50% Iberian-Duroc crossbreeds (farm 3) and using *montanera* periods of different durations (68, 84, or 120 days). The values obtained were similar to those previously reported in the subcutaneous fat of pigs fed during *montanera* (López-Bascón et al., 2015).

The most negative $\delta^{13}C$ (‰) values obtained corresponded to the

Table 1
Average values of $\delta^{13}C$ (‰) according to the characteristics of the subcutaneous fat of biopsies (before the *montanera*) and subcutaneous fat (after the *montanera*).

		100% Iberian		50% Iberian
		Farm 1	Farm 2	Farm 3
Biopsies	Range	−25.18 to −25.59	−25.94 to −26.35	−24.59 to −25.40
	Mean	$-25.37^{b,x}$	$-26.19^{c,x}$	$-25.01^{a,x}$
	N	8	8	8
Sucutaneous fat	Range	−25.35 to −26.63	−26.09 to −26.89	−24.68 to −27.46
	Mean	$-25.89^{a,y}$	$-26.50^{b,y}$	$-26.34^{a,b,y}$
	Days in *montanera*	68	84	120
	N	30	31	45

a,b,c Different letter means statistically significant ($P < 0.05$) differences among groups.
x,y Different letter means statistically significant ($P < 0.05$) differences due to the *montanera*.

biopsied samples taken from 100% Iberian pigs, showing differences that were statistically significant ($P < 0.001$). Previous studies have found that Iberian pigs present higher absolute $\delta^{13}C$ (‰) values than white breeds, which is probably due to the higher intramuscular fat of the Iberian breed (González-Martín et al., 2001). Moreover, two interesting aspects were observed in the subcutaneous fat samples (obtained after slaughter) of the animals that underwent different *montanera* periods. First, a significant increase in the absolute $\delta^{13}C$ (‰) value was observed in 50% Iberian-Duroc crossbreeds ($P < 0.001$) as compared to that obtained using the biopsied samples (taken before *montanera*). This breed of animals were allowed to feed freely during the longest *montanera* period (120 days), with the results indicating that feeding on acorns and grass (C3 plants) significantly influenced the 13C / 12C isotopic ratio. Another relevant finding was that the animals from Farm 2 (100% Iberian) maintained the highest absolute $\delta^{13}C$ (‰) value despite its lower variation compared to the value recorded in the biopsies due to the short period spent in the *montanera*.

These results confirm that the $\delta^{13}C$ (‰) absolute value increased with acorn consumption, as previously reported (González-Martin et al., 1999), and suggest that $\delta^{13}C$ (‰) values could be used to recreate the diet fed to the animals during the *montanera* period. Thus, when the feeding regimes, as established by Spanish legislation (*Bellota* [*montanera* feeding regime], *Cebo de campo* [*montanera* feeding supplemented with fodder] and *Cebo* [fodder feeding]) were compared, the $\delta^{13}C$ (‰) was significantly modified in all cases (López-Bascón et al., 2015). However, no previous studies have been reported on the influence of the *montanera* period on this parameter. As previously mentioned, the different batches of pigs were feed in the same geographical area. On the other hand, breed has not been previously reported to affect the isotopic value $\delta^{13}C$ (‰). Therefore, the observed changes may be associated with the different time of *montanera*.

3.2. Fatty acid composition

The fatty acid compositions of the pigs reared on the three farms before and after *montanera* are shown in Table 2. It should be noted that it was possible to quantify 33 fatty acids from C12:0 to C22:6 n3 using gas chromatography. Additionally, Σsaturated, Σmonounsaturated and Σpolyunsaturated fatty acids and total w3 and w6 were calculated. The results obtained on the 3 farms showed that the *montanera*-based feeding significantly increased the amounts of oleic (C18:1), gadoleic (C20:1 n9), linoleic (C18:2 n-6) and eicosapentaenoic (C20:5 n3) acids. A reduction in saturated acids, such as palmitic acid (C16:0) and stearic acid (C18:0) among others, was also observed. Fatty acid profile modification was especially significant in farm 3 (50% Iberian), the one with the largest *montanera* period, a change that is characteristic of the *montanera* feeding system (Daza et al., 2007; López-Bascón et al., 2015). The significant interaction observed between *montanera* and farm for most of the fatty acids can be attributed to this result. This is because acorns are high in oleic and linoleic acid (>60% and > 17% of total fatty acids respectively) while the main fatty acid found in grass is linolenic acid (> 55%) (Tejerina, García-Torres, Cabeza de Vaca, Vázquez, & Cava, 2011), which is the precursor of the other w3 fatty acids. Furthermore, fatty acid composition of fat in monocavity animals depends on the fat composition of the diet (Monahan, Buckley, Morrissey, Lynch, & Gray, 1992).

Regarding the effect of the breed of the animals, the biopsies from 100% Iberian pigs showed significantly higher levels of C18:1, C18:1n-7 and C18:2 n6, C18:3 n6 ($P < 0.001$). Previous studies have shown that, under similar feeding regimes, purebred Iberian pigs tend to accumulate higher levels of monounsaturated fatty acids, particularly oleic acid, than 50% Iberian x Duroc crosses (Niñoles, Clemente, Ventanas, & Benedito, 2007), as well as higher PUFA contents (Antequera et al., 1994). However, the results of the fatty acid profiles of subcutaneous fat showed that samples from the crossbreeds had higher levels of C18:1, C18:2 n6 and C18:3 n3 acids and lower levels of C16:0 and C18:0 ($P < 0.001$). This result could be associated with the longer *montanera* period, as mentioned above, and justify the significant interaction observed between breed and *montanera* time. This suggests that the feeding regime had a greater influence than the breed of the animals on the fatty acid profile, which is in line with the results of previous studies (De Smet, Raes, & Demeyer, 2004).

3.3. Correlation between fatty acids and $\delta^{13}C$ (‰) values

As previously mentioned, feeding on grass and acorn increases both some fatty acids, such as oleic, linoleic and α-linolenic acids (Daza et al., 2007), and the absolute $\delta^{13}C$ (‰) value in the fat of Iberian pigs (González-Martin et al., 1999). Therefore, it would be interesting to establish whether these correlations could also be found in fat before the *montanera* and if the correlations are stronger after the *montanera*. Furthermore, it is important to determine whether other minor fatty acids were also correlated with $\delta^{13}C$ (‰), as some fodder feeds attempt to mimic the fatty acid profile of the *montanera* feeding albeit only for major fatty acids (Delgado-Chavero et al., 2013). A better understanding of the impact of animal feed on fat composition can help to detect fraud.

Correlation between fatty acid composition and $\delta^{13}C$ (‰) was studied using Pearson's correlation coefficient; the results can be seen in Table 3. A positive correlation (i.e., the lower the absolute value of $\delta^{13}C$ (‰) the higher the fatty acid concentration) could be observed for palmitic and stearic acids, and hence for total saturated fatty acids (SFA), and for γ-linolenic acid (C18:3 n6), both before and after *montanera*. It is noteworthy that after *montanera*, Pearson correlation values were slightly lower. Fodder consumed by pigs is usually rich in saturated fatty acids, which accumulate in adipose tissue, and these samples also showed lower absolute $\delta^{13}C$ (‰) values. On the other hand, negative correlations with $\delta^{13}C$ (‰) were observed for oleic fatty acid (C18:1) and thus with total MUFA, as well as for α-linolenic acid (C18:3 n3) and docosadienoic acid (C22:2). This could indicate a pre-*montanera* feeding based on fodder rich in oleic acid and α-linolenic acid C18:3 n3 that simulates *montanera* feeding. Negative correlations were also found for three fatty acids with an odd number of carbons (C15:0, C17:0 and C17:1) that disappeared or were minimized after the *montanera*, so they were characteristic of fodder-based feeding. (See Table 4.)

After the *montanera*, new negative correlations appeared with linoleic acid (C18:2 n6), erucic acid (C22:1) or arachidonic acid (C20:4 n6). New positive correlations were observed with C20:2. Correlations with some saturated fatty acids disappeared (C20:0) or new positive ones appeared (C23:0). The new negative correlations observed for the sum of PUFA and total n-3 PUFA were remarkable. It should be recalled that after the *montanera* the more negative the value of the isotopic ratio the

Table 2
Composition of fatty acids (%) of the 3 farms in subcutaneous fat of biopsies (before the *montanera*) and in subcutaneous fat (after the *montanera*).

Fatty acids	100% Iberian								50% Iberian								
	FARM 1				FARM 2				FARM 3								
	Before M		After M		Before M		After M		Before M		After M		*P*-value				
	n = 8		n = 31		n = 8		n = 30		n = 8		n = 45						
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Breed	M	Farm	B*M	M*F
C12:0	0.06	0.003	0.05	0.006	0.07	0.004	0.06	0.005	0.09	0.009	0.06	0.008	<0.001	<0.001	<0.001	<0.001	<0.001
C14:0	1.17	0.029	1.10	0.097	1.22	0.057	1.20	0.081	1.21	0.059	1.04	0.108	0.007	<0.001	<0.001	<0.001	<0.001
C14:1 n5	0.00	<0.001	0.01	0.017	0.01	0.008	0.01	0.008	0.00	0.008	0.01	0.014	0.379	0.116	<0.001	0.853	0.699
C15:0	0.05	0.004	0.04	0.007	0.06	0.004	0.05	0.018	0.05	0.003	0.04	0.020	0.077	0.011	0.002	0.299	0.138
C16:0	21.54	0.310	20.20	0.692	19.80	0.458	19.50	0.919	20.82	0.292	17.55	1.148	<0.001	<0.001	<0.001	<0.001	<0.001
C16:1	2.38	0.097	1.95	0.192	2.65	0.112	2.47	0.195	2.45	0.215	2.10	0.200	0.080	<0.001	<0.001	0.710	0.006
C17:0	0.34	0.021	0.28	0.050	0.35	0.024	0.29	0.039	0.27	0.020	0.26	0.053	<0.001	<0.001	<0.001	0.002	0.010
C17:1	0.32	0.027	0.26	0.045	0.36	0.023	0.32	0.038	0.26	0.018	0.25	0.060	<0.001	0.001	<0.001	0.041	0.031
C18:0	11.80	0.361	10.28	0.895	8.73	0.393	8.17	0.837	10.50	0.625	7.59	0.724	<0.001	<0.001	<0.001	<0.001	<0.001
C18:1 n9	0.17	0.155	0.01	0.054	0.06	0.119	0.00	<0.001	0.06	0.106	0.00	0.009	0.012	<0.001	<0.001	0.044	<0.001
C18:1	47.10	0.643	52.11	1.016	49.41	0.770	53.08	1.022	46.64	0.542	55.63	1.495	0.001	<0.001	<0.001	<0.001	<0.001
C18:1 n7	2.84	0.306	1.87	0.286	2.74	0.296	2.10	0.295	2.71	0.294	1.67	0.423	0.001	<0.001	0.003	0.067	0.010
C18:2 n6t	0.12	0.005	0.10	0.015	0.14	0.007	0.13	0.014	0.12	0.013	0.10	0.021	0.001	<0.001	<0.001	0.279	0.004
C18:2 n6	8.36	0.237	8.04	0.376	10.47	0.227	8.67	0.675	11.08	0.453	9.70	0.901	<0.001	<0.001	<0.001	0.209	<0.001
C18:3 n6	0.14	0.006	0.10	0.021	0.15	0.012	0.15	0.027	0.12	0.010	0.10	0.020	<0.001	<0.001	<0.001	0.651	<0.001
C18:3 n3	0.22	0.017	0.22	0.025	0.17	0.012	0.08	0.063	0.20	0.012	0.17	0.019	0.404	0.002	<0.001	0.482	<0.001
C20:0	2.03	0.091	0.59	0.077	2.15	0.112	0.70	0.092	1.96	0.078	0.83	0.155	0.212	<0.001	<0.001	<0.001	<0.001
C20:1 n9	0.07	0.005	1.58	0.189	0.06	0.010	1.57	0.164	0.06	0.006	1.52	0.206	0.319	<0.001	0.581	0.374	0.668
C21:0	0.11	0.033	0.07	0.028	0.09	0.016	0.14	0.014	0.11	0.011	0.13	0.017	0.010	0.030	<0.001	0.088	<0.001
C20:2 n6	0.05	0.005	0.03	0.013	0.04	0.013	0.02	0.008	0.03	0.013	0.02	0.008	<0.001	<0.001	<0.001	<0.001	<0.001
C22:0	0.03	0.012	0.02	0.009	0.04	0.012	0.03	0.014	0.05	0.004	0.02	0.009	0.039	<0.001	<0.001	<0.001	<0.001
C20:3 n6	0.00	<0.001	0.03	0.007	0.00	<0.001	0.03	0.006	0.00	<0.001	0.02	0.006	<0.001	<0.001	<0.001	<0.001	<0.001
C22:1 n9	0.57	0.024	0.57	0.051	0.64	0.035	0.66	0.074	0.68	0.027	0.64	0.071	<0.001	0.193	<0.001	0.133	0.192
C20:3 n3	0.03	0.012	0.03	0.004	0.03	0.011	0.03	0.007	0.02	0.010	0.03	0.006	0.001	<0.001	<0.001	0.249	0.508
C20:4 n6	0.09	0.004	0.01	0.007	0.10	0.008	0.08	0.015	0.10	0.007	0.01	0.011	0.001	<0.001	<0.001	<0.001	<0.001
C23:0	0.01	0.009	0.09	0.010	0.02	0.008	0.03	0.005	0.01	0.009	0.08	0.010	0.010	<0.001	<0.001	0.012	<0.001
C22:2 n6	0.16	0.014	0.01	0.008	0.25	0.018	0.22	0.031	0.19	0.013	0.02	0.006	<0.001	<0.001	<0.001	0.002	<0.001
C20:5 n3	0.01	0.009	0.15	0.018	0.00	0.001	0.09	0.043	0.00	0.007	0.21	0.030	<0.001	<0.001	<0.001	<0.001	<0.001
C22:5 n3	0.14	0.007	0.13	0.017	0.13	0.011	0.04	0.037	0.14	0.013	0.13	0.022	<0.001	<0.001	<0.001	0.019	<0.001
C22:6 n3	0.09	0.007	0.07	0.007	0.07	0.004	0.07	0.012	0.07	0.006	0.06	0.009	<0.001	<0.001	<0.001	0.641	<0.001
AGS	35.26	0.492	32.24	1.283	30.53	0.655	29.62	1.652	33.22	0.634	26.87	1.796	<0.001	<0.001	<0.001	<0.001	<0.001
AGM	53.44	0.468	58.34	1.209	55.94	0.694	60.21	1.267	52.87	0.712	61.83	1.244	0.111	<0.001	<0.001	<0.001	<0.001
AGP	11.30	0.163	9.42	0.390	13.53	0.225	10.17	0.780	13.91	0.472	11.29	1.041	<0.001	<0.001	<0.001	0.928	<0.001
w3	2.30	0.095	0.97	0.078	2.38	0.107	0.93	0.119	2.20	0.073	1.25	0.179	0.001	<0.001	0.003	<0.001	<0.001
w6	9.00	0.222	8.45	0.366	11.15	0.217	9.24	0.701	11.71	0.449	10.04	0.881	<0.001	<0.001	<0.001	0.101	<0.001

M = *montanera,* B = Breed, F = Farm, sd = standard deviation.

Table 3
Correlation between the composition of $\delta^{13}C$ (‰) and the fatty acids and in subcutaneous fat of biopsies (before the *montanera*) and in subcutaneous fat after the *montanera*.

	Biopsies (Before the *montanera*)		Subcutaneus fat (After the *montanera*)	
Fatty acid	Pearson correlation with $\delta^{13}C$ (‰)	*P*-value	Pearson correlation with $\delta^{13}C$ (‰)	*P*-value
C16:0	0.656	0.001	0.563	<0.001
C18:0	0.645	0.001	0.570	<0.001
C18:1	−0.816	<0.001	−0.530	<0.001
C18:2 n6	−0.054	0.804	−0.600	<0.001
C18:3 n6	0.657	<0.001	0.459	<0.001
C18:3 n3	−0.621	0.001	−0.588	<0.001
C20:1 n9	0.282	0.183	0.238	0.010
C21:0	0.167	0.434	−0.364	<0.001
C20:2 n:6	−0.264	0.213	0.217	0.030
C22:1 n9	0.049	0.819	−0.307	<0.001
C20:4 n6	−0.058	0.789	−0.343	<0.001
C23:0	−0.096	0.656	0.354	<0.001
C22:2 n6	−0.725	<0.001	−0.336	<0.001
C22:5 n3	0.180	0.400	0.220	0.020
C22:6 n3	0.275	0.193	0.307	<0.001
SFA	0.670	<0.001	0.600	<0.001
MUFA	−0.817	<0.001	−0.509	<0.001
PUFA	−0.152	0.480	−0.606	<0.001
n-6	−0.595	0.002	−0.389	<0.001
n-3	−0.092	0.669	−0.629	<0.001

higher the correlation with feeding based on C3 plants such as acorns that contain a significant amount of C18:2 n6 (>17%) and grass that is rich in n-3 PUFA (>57%) (Tejerina et al., 2011). These new correlations pointed out that the most negative values of $\delta^{13}C$ (‰) (obtained from the samples of animals that have spent more time in *montanera*) were related to these unsaturated fatty acids, responsible for the quality of Iberian products.

3.4. Discriminant analysis

First, a discriminant analysis was performed using only NIR spectral information. A wavelength range of 1100 to 2000 nm was used, and the differentiation method used was RMS X Residual. The square root of the mean squared residual (RMS) was calculated to obtain the RMS X residual. The mathematical treatment applied was Standard Normal Variate (SNV) (2,4,4,1), where the first digit is the order of the derivative, the second is the gap over which the derivative is calculated, the third is the number of data points of a continuous or smoothed average, and finally the fourth is the second smoothing. Fat samples from the biopsies before *montanera* (8 samples per farm representing 24 animals sampled) and subcutaneous fat samples after *montanera* (30 samples from Farm 1, 31 samples from Farm 2 and 45 samples from Farm 3) were analyzed using the cams-locks. Using only the spectral information, the three fat groups, originating from the biopsied tissue, was successfully differentiated in all samples analyzed (i.e. 100%). Similarly, the three groups of extracted subcutaneous fat were also successfully discriminated. These results are similar to those reported by García-Olmo, Garrido-Varo, and De Pedro (2009) in their study on extracted fat, where different acorn feeding times (30, 60 and 180 days) were discriminated. This 100% accuracy can be attributed to the fact that subcutaneous fat spectra reflect changes in feeding regimes as they directly affect the fatty acid profile. Moreover, the extraction process allowed for greater homogeneity, avoiding the interference of water and thus improving the ability to differentiate. However, the percentages reported for direct differentiation of commercial categories or feeding regimes (acorn vs. concentrate) in subcutaneous fat were slightly lower (74 and 93%, respectively) (Horcada, Valera, Juárez, & Fernández-Cabanás, 2020).

Differentiation of the samples collected from the three farms was then carried out using $\delta^{13}C$ (‰) and fatty acid data. In this case, the screen was carried out using the subcutaneous fat samples and the whole collection of samples, without distinguishing whether they were taken

Table 4
Percentage of samples classified in accordance with their origin for model OPLS-DA for the samples of subcutaneous fat from subcutaneous fat after the *montanera* and for biopsies and subcutaneous fat as a whole for both internal and external validation.

Subcutaneous fat	Internal validation					
		Members	Correct	Farm 1	Farm 2	Farm 3	No class
	Farm 1	25	100%	25	0	0	0
	Farm 2	24	100%	0	24	0	0
	Farm 3	35	100%	0	0	35	0
	No class	0		0	0	0	0
	Total	84	100%	25	24	35	
	External validation						
		Members	Correct	Farm 1	Farm 2	Farm 3	No class
	Farm 1	6	100%	6	0	0	0
	Farm 2	6	100%	0	6	0	0
	Farm 3	6	100%	0	0	9	0
	No class	0		0	0	0	0
	Total	21	100%	6	6	9	
Subcutaneous fat-biopsies	Internal validation						
		Members	Correct	Farm 1	Farm 2	Farm 3	No class
	Farm 1	32	100%	32	0	0	0
	Farm 2	32	100%	0	30	0	0
	Farm 3	41	100%	0	0	41	0
	No class	0		0	0	0	0
	Total	103	100%	32	30	41	
	External validation						
		Members	Correct	Farm 1	Farm 2	Farm 3	No class
	Farm 1	7	100%	7	0	0	0
	Farm 2	8	100%	0	8	0	0
	Farm 3	11	100%	0	0	11	0
	No class	0		0	0	0	0
	Total	26	100%	7	8	11	

before (biopsies) or after the *montanera*. First, potential outliers were detected. For this purpose, an individual PCA was applied for each farm with a 95% confidence interval. One outlier was detected on Farm 3 and excluded for the subsequent analysis.

Subsequently, OPLS-DA was applied to the different datasets (106 subcutaneous fat samples and 130 subcutaneous fat and biopsy samples) using $\delta^{13}C$ (‰) and fatty acid values for classification. Using this data, chemometric models were built using 80% of the samples for the training set and the remaining 20% for the validation set.

The first model (Fig. 1a) corresponded to the 106 samples taken from the subcutaneous fat at the time of slaughter. The model created successfully classified all of the categories in the external and internal validation. Then, a model was built for all samples considering both sample types (biopsies and subcutaneous fat) together (Fig. 1b), giving a total of 129 samples. Again, the classification of the samples, for internal and external validation, was 100% correct. The fatty acids with the highest potency, with a p.corr greater than or close to 1, were: stearic (C18:0); docosadienoic (C22:2); palmitic (C16:0); linoleic (C18:2 n6); γ-linolenic (C18: 3 n:6); heneicosanoic (C21:0); DPA (C22:5 n6); eicosapentaenoic (C20:5 n3); palmitoleic (C16:1); and oleic acid (C18:1); and a $\delta^{13}C$ (‰) value greater than 9 (Fig. 2).

Correct classification was also obtained in 100% of the cases during both external and internal validation when only fatty acid values were used. However, Q2 values (which is an estimation of the predictive potential of the model) were lower than those obtained when isotopes were included in the differentiation model. This difference was observed for the differentiation models for subcutaneous fat (0.886 vs. 0.895) and for subcutaneous fat and biopsies together (0.842 vs. 0.886). This result reveals the important contribution of the isotope ratio for sample differentiation according to the duration of the *montanera* period. These results show that the statistical combination of fatty acid and $\delta^{13}C$ (‰) data could be a way to discriminate Iberian pork samples.

Nevertheless, previous studies have shown that it is possible to discriminate subcutaneous fat samples according to feed type (acorn only, acorn plus pre-mixed feed, or pre-mixed feed) with 90% accuracy. In this case fatty acids and $\delta^{13}C$ were also used (‰) but the PLS-DA algorithm was applied instead of OPLS-DA. Moreover, all of the models showed that $\delta^{13}C$ (‰) had the best predictive performance (López-Bascón et al., 2015). This is consistent with the improved predictive ability observed when $\delta^{13}C$ (‰) was included in the models.

Although there are several studies that aim to classify the Iberian pig according to its feeding regime (*bellota* - fed with acorns and grass during

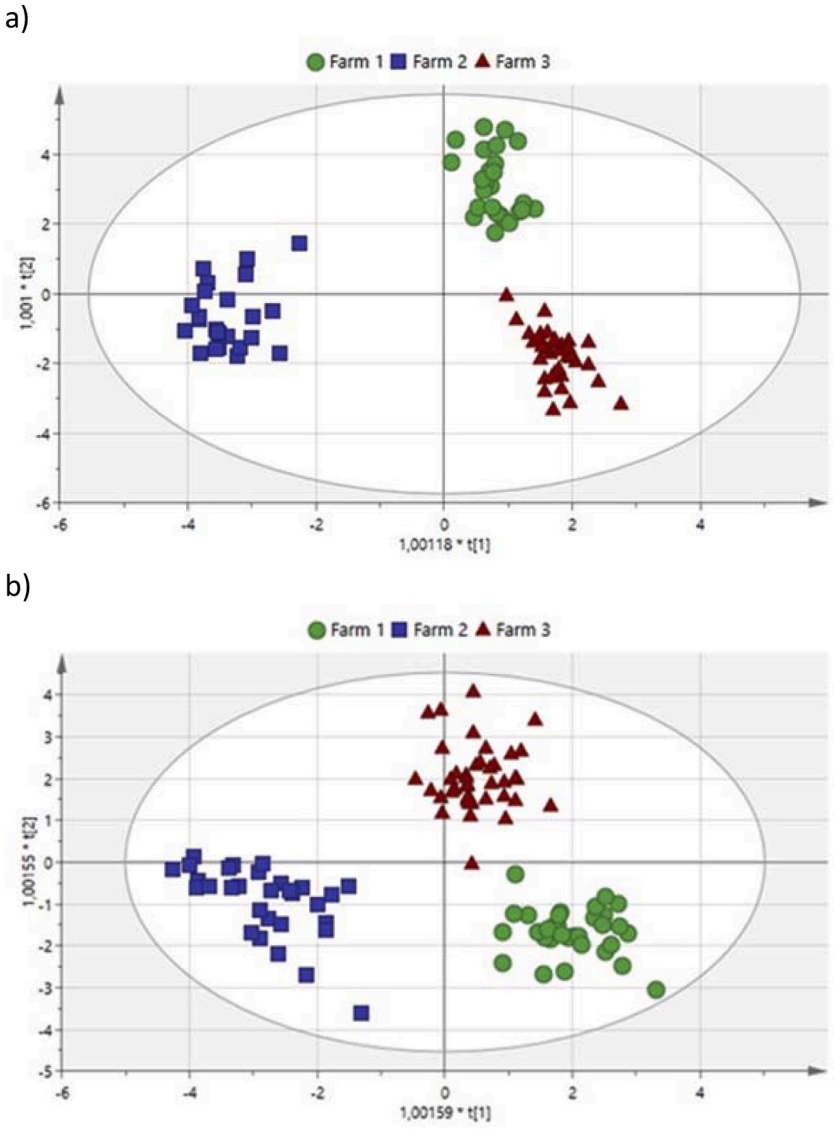

Fig. 1. Developed OPLS-DA models for a) subcutaneous fat of biopsies (before the *montanera*), b) subcutaneous fat after the *montanera*, c) biopsies and subcutaneous fat.

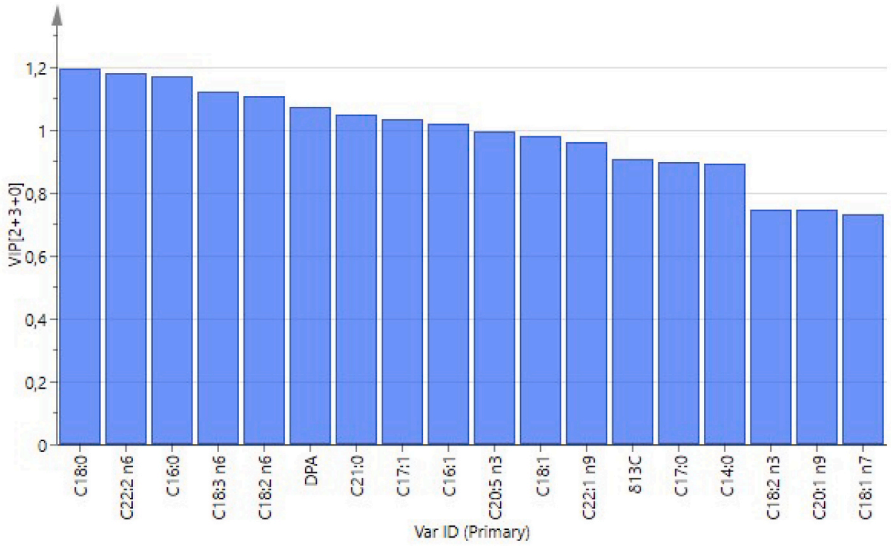

Fig. 2. The most important and significant variables for model OPLS-DA of subcutaneous fat of biopsies (before the *montanera*) and subcutaneous fat after the *montanera* as a whole.

the *montanera; recebo* - fed with natural food but fed formulated feed close to slaughter; and *cebo* - fed only with formulated feed) (Delgado-Chavero et al., 2013; López-Bascón et al., 2015; Recio et al., 2013; Ruiz et al., 1998), currently there are no studies that have attempted to distinguish different periods of *montanera*.

3.5. $\delta^{13}C$ (‰) and fatty acid calibration equations

3.5.1. Calibration equations

Table 5 shows the $\delta^{13}C$ (‰) and fatty acid calibration descriptors together for the whole sample set (biopsies collected before the *montanera* and subcutaneous fat collected after the *montanera* after the animals were slaughtered; 104 samples in total). This table shows the number of samples (N) used in the model after some samples were removed based on spectral (H) or chemical (T) criteria, as well as the results of the descriptors applying the best mathematical treatment.

The NIRS equations allow for the determination of the following $\delta^{13}C$ (‰) and 14 fatty acids in extracted fat samples: palmitic acid, C16:0; palmitoleic acid, C16:1; heptadecanoic acid, C17:0; cis-10 heptadecenoic acid, C17:1; stearic acid, C18:0; oleic acid, C18 1; trans-vaccenic acid, 18: 1 n7; linoleic acid, C18:2; γ-linolenic acid, C18:3 n6; α-linolenic acid, C18:3 n3; eicosenoic acid, C20:1; eicosapentaenoic acid, C20:5 n3; heneicosanoic acid, C21:0; erucic acid, C22:1; docosadienoic acid, C22:2 n6; and DPA, C22:5 n3. Furthermore, it is also possible to determine five fatty acid sums: Σ of saturated fatty acids (SFA), Σ of monounsaturated fatty acids (MUFA) and Σ of polyunsaturated fatty acids (PUFA) together with Σ of PUFA n3 and Σ of PUFA n6. The NIR calibration descriptors shown in Table 5 were found to be satisfactory, with RSQ > 0.8 for almost all of the calibrated parameters. It is remarkable that the application ranges and standard deviations of these mathematical models for the determination of $\delta^{13}C$ (‰) and fatty acids is similar and comparable to the results obtained for $\delta^{13}C$ (‰) by isotope ratio mass spectrometry (IRMS) (Table 1) and for fatty acids by gas chromatography (Table 2). Therefore, they have useful statistical values when applying the models to unknown samples.

A novel approach is the determination of the 13C/12C isotope ratio expressed as $\delta^{13}C$ (‰) using NIRS, especially considering the importance of this parameter and the economic cost for potential users. Although the

Table 5
NIR calibration statistics of fatty acids and stable carbon isotopes in Iberian pig biopsies and subcutaneous fat.

Constituent	N	Mean	Est. Min	Est. Max	SD	SEC	SEP	RSQ
δ13C	99	26.17	24.71	27.62	0.48	0.21	0.40	0.81
C16:0	101	19.19	14.52	23.86	1.56	0.33	1.05	0.96
C16:1	102	2.21	1.35	3.06	0.28	0.14	0.24	0.77
C17:1	101	0.28	0.12	0.43	0.05	0.03	0.05	0.59
C18:0	99	8.89	4.32	13.47	1.53	0.42	0.78	0.92
C18:1	98	53.11	44.66	61.57	2.82	0.83	1.69	0.91
C18:1 n7	99	19.84	6.10	33.59	4.58	2.30	3.84	0.75
C18:2 n6	100	9.01	5.94	12.07	1.02	0.34	0.87	0.89
C18:3 n3	99	0.92	0.00	2.43	0.50	0.20	0.35	0.83
C18:3 n6	102	0.17	0.00	0.35	0.06	0.03	0.06	0.81
C20:1 n9	98	1.34	0.00	2.98	0.55	0.21	0.48	0.85
C20:5 n3	84	0.16	0.00	0.32	0.05	0.01	0.04	0.96
C21:0	102	0.11	0.01	0.22	0.03	0.02	0.03	0.69
C22:1 n9	101	0.62	0.43	0.82	0.06	0.04	0.05	0.69
C22:2 n6	97	0.10	0.00	0.39	0.10	0.04	0.09	0.83
SFA	99	29.95	21.00	38.89	2.98	0.56	1.58	0.97
PUFA	100	10.76	6.65	14.86	1.37	0.43	1.18	0.90
MUFA	98	59.55	51.23	67.86	2.77	0.81	1.63	0.91
n3	98	1.26	0.00	2.70	0.48	0.21	0.35	0.81
n6	100	9.50	6.35	12.65	1.05	0.28	0.87	0.93
δ13C	99	26.17	24.71	27.62	0.48	0.21	0.40	0.81

N = number of samples worked. SEC = Standard calibration error. SD = standard deviation. RSQ = correlation coefficient. SEP = Standard prediction error.

determination of the major fatty acids C16:0, C18:0, C18:1, C18:2, and C20:1, SFAs, MUFAs, and PUFAs by NIR spectroscopy has been previously reported (De Pedro et al., 1992, 1995; Fernández-Cabanás et al., 2007; Gonzalez-Martin et al., 2003; Pérez-Juan et al., 2010; Zamora-Rojas, Garrido-Varo, De Pedro-Sanz, Guerrero-Ginel, & Pérez-Marín, 2013), this technique has not been used to quantify the minor fatty acids included in this study. Furthermore, the calibration equations can be applied to determine these compounds in the extracted fat samples collected at different times during the pig fattening process.

The NIR wavelength range is associated with combinations of C—H stretching with other vibrational modes (Westad, Schmidt, & Kermit, 2008). Thus, the C—H bond, which is a fundamental constituent of fatty

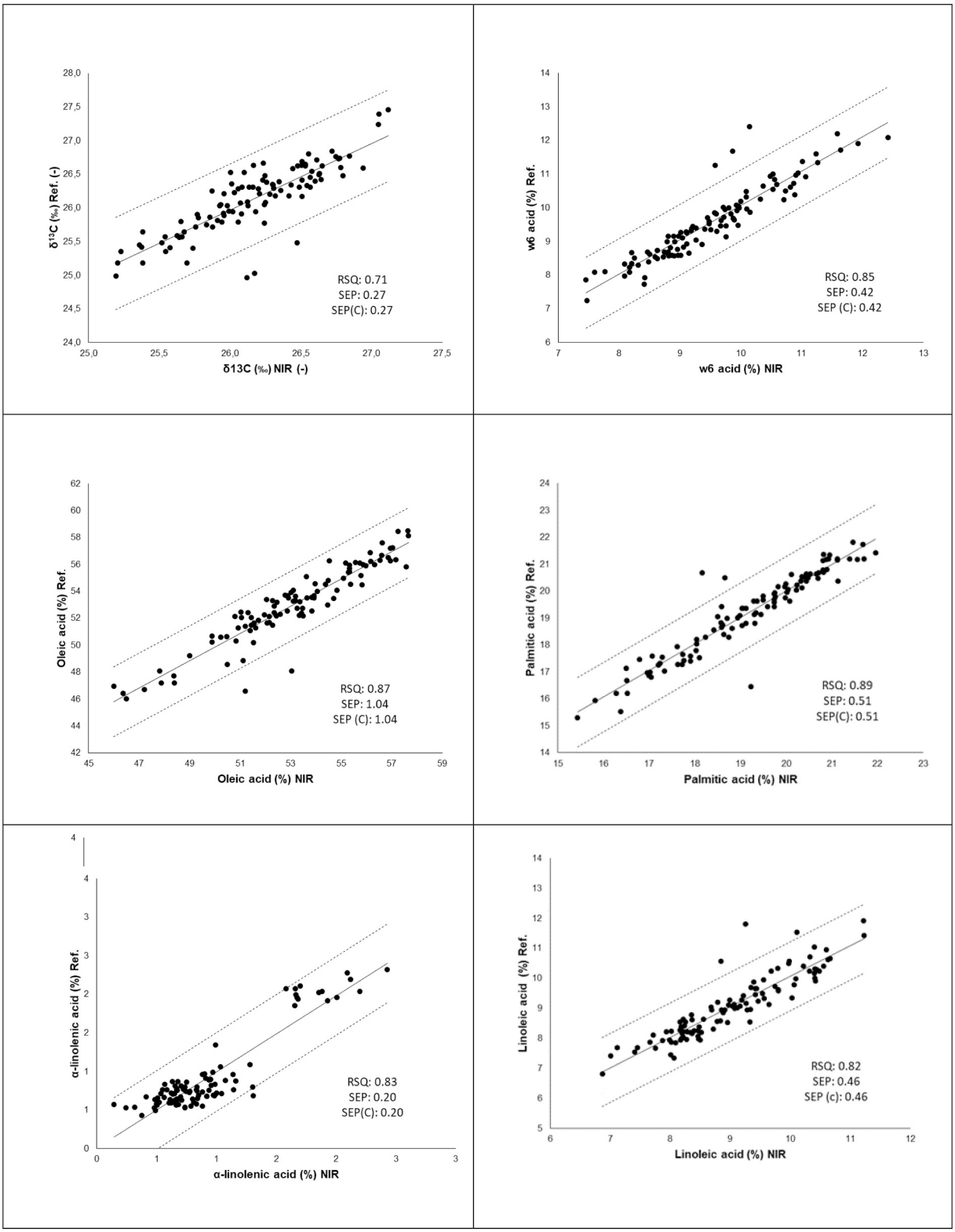

Fig. 3. Internal validation. Comparative study of $\delta^{13}C$ and fatty acids.

acid molecules, is strongly absorbs at wavelengths near 1200, 1400, 1750, 2310 and 2340 nm (Williams & Norris, 1987). Furthermore, the 2310–2340 region corresponds to the C—H bond combination bands and the absorption produced in the 1720–1760 region corresponds to the first overtone of that bond (Gonzalez-Martin et al., 2003; Shenk, Westerhaus, & Workman, 1992; Zamora-Rojas et al., 2013). Some authors attributed the absorption at 1210 nm to the second overtone of the CH_2 bond (Osborne, Fearn, & Hindle, 1993). Absorption in the region of 2150–2190 and at 1680 nm indicates the presence of cis-double bonds, i.e., unsaturated fatty acids (Garrido-Varo, Carrete, & Fernández-Cabanás, 1998). Regarding δ13C (‰), the regression model can be justified using the correlation between concentration and measurement at different wavelengths in accordance to the equation $y = \beta_0 + \beta_1 X_{\lambda 1} + \beta_2 X_{\lambda 2} + \beta_3 X_{\lambda 3} + \ldots\ldots + \beta_n X_{\lambda n}$, where β are the coefficients and $X_{\lambda 1}, X_{\lambda 2}, X_{\lambda 3}, \ldots\ldots\ldots X_{\lambda n}$, are the wavelengths where there is correlation. $\delta^{13}C$ (‰) has β values of 377.3 at 1720 nm (corresponding to the C-0 band of the oil) and 197.9 to 1948 nm (corresponding to the presence of C═O stretching bands, a second CO2R overtone) among others.

3.5.2. Internal and external validation

NIR calibration models were internally assessed by means of cross-validation. In this method, the sample calibration set is divided into a number of subsets, which in this study is seven. Of the latter, six were used for the calibration set and one for the prediction set. The NIRS calibration model using MPLS allows for the determination of $\delta^{13}C$ (‰) and fatty acids in subcutaneous fat (biopsies before *montanera* and subcutaneous fat after *montanera*) by recording NIR spectra in melted fat in cam-lock cells. Table 5 shows the standard error of prediction (SEP) of the models for which the difference between SEP and the standard error of calibration (SEC) was lower and the RSQ value was higher. Comparison of the NIRS data obtained using the mathematical models and the reference data for $\delta^{13}C$ (‰) and some fatty acids are shown in Fig. 3. It can be seen that the validation RSQ values were higher than 0.8 and the SEP and SEP (C) values were similar. The RSQ values observed for both the calibration and validation of palmitic, stearic, linoleic and linolenic acids were either similar to those previously reported in the subcutaneous fat of Iberian pigs (Gonzalez-Martin et al., 2003; Pérez-Juan et al., 2010; Zamora-Rojas et al., 2013) or higher, like the value obtained for C18:3 n3 (González-Martín et al., 2021).

The robustness of the method was confirmed by using NIRS technology to analyze 26 new samples of subcutaneous fat (from the biopsies collected before *montanera* and the subcutaneous fat collected after *montanera*). Student's *t*-test analysis for paired values compared the $\delta^{13}C$ (‰) and fatty acid values obtained with the reference methods (mass spectrometry and chromatography) with those predicted in the external validation with the NIRS model. The *P*-values obtained were above the minimum level of significance (0.05). Therefore, the null hypothesis is accepted and it can be stated that there is no difference between the $\delta^{13}C$ (‰) and fatty acid values generated using the different methods. From these results it can be concluded that the NIRS technique is a useful alternative to isotopic mass spectrometry and gas chromatography for the determination of all the parameters studied in subcutaneous fat samples.

4. Conclusion

Using only NIR spectra or fatty acid and stable isotopes data, it was possible to distinguish between farms employing different feeding regimes (length of the *montanera* period), with 100% of the samples being classified correctly. Moreover, the differentiation was possible in both samples types: fat extracted from biopsied tissue (collected before *montanera*) and subcutaneous fat taken from pig carcasses (after *montanera*). Also, using NIR spectroscopy, it was possible to calibrate 14 fatty acids, the sums of the main fatty acid groups and the $\delta^{13}C$ ratio (‰). Among the fatty acids that could be calibrated were those that had a major contribution to the OPLS-DA model for sample differentiation on a farm-by-farm basis. Also, it was possible to calibrate most of that fatty acids showing a higher correlation with $\delta^{13}C$ (‰), or with quality Iberian products, such as C18:1, C18:2 and C18:3. These results prove that NIR spectroscopy, together with stable carbon isotopes, is a powerful technique that can be used to differentiate Iberian pork samples. Thus, the Iberian pork industry could use NIR spectroscopy as a routine tool to analyze subcutaneous fat at slaughter. NIRS would allow $\delta^{13}C$ (‰) to be determined and correlated with the diet during the *montanera* as well as the fatty acids responsible for the production of quality meat.

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Declaration of Competing Interest

None.

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