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^{1}H NMR to analyse the lipid profile in the glyceride fraction of different categories of Iberian dry-cured hams

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ABSTRACT

The extraordinary organoleptic qualities of Iberian ham derive from the singular producing pig breed and from the traditional rearing conditions, both of which define its lipid content and composition. In this work ^{1}H NMR spectroscopy is used for the first time to analyse the lipid profile of Iberian hams as determinant of quality. Quantification of fatty acids is readily obtained from the spectra, with the monounsaturated fatty acids standing out, especially in the higher quality hams. Unprecedently, triacylglyceride hydrolysis products formed during the curing process can also be directly detected and quantified. Furthermore, chemometric analysis of the NMR data allows to classify Iberian hams according to the pig's crossbreed and feeding regime. Principal component analysis shows fatty acid unsaturation and triacylglyceride hydrolysis as discriminating variables. ^{1}H NMR spectroscopy has thus revealed as a convenient and powerful tool for the lipid analysis and classification of Iberian hams and for detection of fraud.

1. Introduction

Iberian dry-cured ham is a gastronomic delicacy traditionally produced from Iberian pigs, a native breed from the southwest of the Iberian Peninsula. The exceptional taste and flavour characteristics of Iberian ham have prompted its worldwide popularity and great commercial value. Several factors, such as crossbreed, rearing system and processing conditions have a fundamental impact on the quality of the final product.

Within the factors included under rearing system, feeding seems to be the most determining factor in Iberian ham quality (Carrapiso, Bonilla, & García, 2003; Cava, Ventanas, Florencio Tejeda, Ruiz, & Antequera, 2000). Iberian hams from animals fed in extensive regime with natural resources reach the highest prices in the market because of their great sensory traits, which are mainly attributed to the consumption of acorns and grass (Pérez Palacios, Ruiz, Tejeda, & Antequera, 2009). However, since these natural resources are seasonal (October-February) and in limited supply, other feed types and feeding practices are also used. In addition, the use of crossbred, mainly Iberian × Duroc pigs, rather than purebred animals is frequently carried out to improve growth rate and lean content in carcass (***Tejeda, Gandemer, Antequera, Viau, & García, 2002). Crossbreeding and/or substitution of acorn and grass by a concentrate diet may lead to a marked decrease in the final nutritional quality of Iberian products in terms of lipid composition (lower levels of unsaturated fatty acids) and sensory attributes (i.e., lower marbling, brightness and flavour intensity, among others).

To avoid commercial fraud and guarantee the rights of consumers, the Spanish Ministry of Agriculture, Fishery and Food enacted a law to standardise the different qualities of Iberian meat products (Real Decreto 4/2014, de 10 de enero, 2014). This establishes four categories for Iberian ham depending on the breed and the feeding system, which are labelled with different colours: "Black", corresponding to 100% Iberian pigs exclusively fed on acorns and grass in an extensive system (montanera); "Red", meat pieces from pigs fed in the same way than "Black" yet at least 50% Iberian breed; "Green", from pigs that are at least 50% Iberian breed, reared in extensive farms with pasture and acorns complemented with concentrate feeds; and "White", from pigs that are at least 50% Iberian breed reared in an intensive system. The crossbreed and rearing conditions during fattening affect the lipid content and fatty acid profile of pig tissues (Pérez Palacios, Ruiz, Tejeda, & Antequera, 2009; Tejeda et al., 2002), which is one of the most

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important characteristics defining the sensorial quality of Iberian ham. In fact, most of the techniques applied to differentiate Iberian pigs according to breed and/or diet are based on their lipid profile. The proportions of major fatty acids (FA), i.e., palmitic (C16:0), stearic (C18:0), oleic (C18:1n-9) and linoleic (C18:2n-6) acids, in subcutaneous fat were used to classify Iberian pigs as a function of their feeding background during fattening (Real Decreto 4/2014, de 10 de enero, 2014). Therefore, a proper quality control and the need to comply with the standard regulations encouraged the development of reliable characterization methods, many of which are based on the analysis of the lipidomic profile.

The most common method for analysing FA in meat relies on gas chromatography with flame ionization detector (GC-FID) with a pre-transmethylation procedure (Petrón, Muriel, Timón, Martín, & Antequera, 2004). Other methods have also been proposed to analyse different categories of Iberian pigs. Thus, the analysis of FA *cis/trans* isomers from subcutaneous fat by GC-FID has been evaluated to classify Iberian pigs according to the rearing system (Sánchez González & Fernández Bermejo, 2007). Alternatively, the determination of $^{13}C/^{12}C$ ratio in the fatty acid methyl esters by means of Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) has been used with the same purpose (Recio, Martín, & Raposo, 2013). Near Infrared Spectroscopy (NIR) has also been applied to differentiate Iberian pigs subjected to different rearing systems (Pérez-Marín, Fearn, Riccioli, De Pedro, & Garrido, 2021). Furthermore, some compounds from the lipidic unsaponifiable fraction, such as neophytadiene (Tejeda, Antequera, Martín, Ventanas, & García, 2001) or tocopherols (Rey, Lopez-Bote, & Sanz Arias, 1997), have been considered as feeding markers in Iberian pigs. In a complementary way, Time Domain Nuclear Magnetic Resonance (TD-NMR), or NMR relaxometry, has been used to check the degree of maturation of ham since this is related to the water retained in the curing process (García-García, Cambero, Castejón, Escudero, & Fernández-Valle, 2019). Likewise, Magnetic Resonance Imaging (MRI) has been evaluated to estimate the quality traits of Iberian hams at different stages of the curing process in a non-destructive way (Caballero et al., 2016).

In recent years, nuclear magnetic resonance (NMR) spectroscopy has emerged as a powerful tool in food analysis, with applications in the identification and quantification of food constituents, determination of metabolomic profiles, food authentication and quality control. Among the many analytes that can be determined by NMR are fats and proteins in caviar, citric acid in oranges, carbohydrates and polyols in vinegar, and free and esterified sterols in olive oil, just to name a few (Cao et al., 2021).

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been widely used for the lipid analysis of different types of foods (Alexandri et al., 2017), including edible oils (Castejón, Mateos-Aparicio, Molero, Cambero, & Herrera, 2014), eggs (Hajjar, Haddad, Rizk, Akoka, & Bejjani, 2021), dairy products (Tsiafoulis et al., 2019), fish (Mannina et al., 2008) and meat (Siciliano et al., 2013). Compared to other analytical methods, NMR provides major advantages, such as minimal sample handling and no need for derivatization, standards or calibration curves for quantitative analysis (Alexandri et al., 2017; Castejón et al., 2014). This reduces the cost of individual analyses and saves time in both instrumental analysis and post-analysis calculations. Importantly, ^{1}H NMR lipid analysis readily provides both qualitative and quantitative information. Thus, simple inspection of the spectrum by a trained person allows to identify diagnostic peaks characteristic of specific compounds. Furthermore, the automation of NMR processing, combined with chemometric approaches, has been used in the authentication and classification of several foodstuffs (Hajjar et al., 2021; Martin-Pastor, Guitian, & Riguera, 2016). However, to the best of our knowledge, ^{1}H NMR spectroscopy has not been used so far to determine the lipid profile of dry-cured meat products, such as loins or hams. As the ^{1}H NMR spectrum reflects the totality of the organic compounds in a sample, NMR analysis of the lipid fraction of a cured ham should give a complete picture of its lipid profile, which is largely determined by the crossbreed pigs, the rearing system, and the technological conditions of the curing process.

Thus, the objective of the present study was 1) to analyse the fatty acid composition and glyceride fraction of the intramuscular lipids from different Iberian dry-cured hams by NMR and 2) to evaluate the ability of NMR spectroscopy to classify Iberian dry-cured hams of different categories.

2. Materials and methods

2.1. Reagents and standards

Chloroform, methanol and sodium sulphate from Scharlau (Barcelona, Spain) were used for the lipid extraction. *n*-Hexane, methanol, metallic sodium, sulphuric acid from Scharlau, sodium chloride from Panreac (Barcelona, Spain), Supelco 37-component FAME mix (Merck) and tridecanoic acid from Sigma (Madrid, Spain) were used in the transesterification procedure. Deuterochloroform ($CDCl_3$) and tetramethylsilane (TMS) from Sigma were used in the NMR analysis.

2.2. Experimental design

Samples from dry-cured hams (sliced in packs of 90 g) were used in this study. Four commercial brands of each category (Black, Red, Green and White) were purchased in a dry-cured Iberian ham industry, making a total of 16 samples. The samples were shredded and three 5 g portions were taken from each sample. All 48 resulting samples were then vacuum packed and frozen until being analysed.

2.3. Lipid extraction

All the samples were subjected to the same process to extract the total intramuscular lipids. Intramuscular fat was extracted following the method of Folch (Folch, Lees, & Stanley, 1957) modified by Pérez-Palacios (Pérez-Palacios, Ruiz, Martín, Muriel, & Antequera, 2008). According to this procedure, 5 g of sample was homogenized with 100 mL of chloroform-methanol (2:1, v:v), using an omni mixer homogenizer. The obtained mixture was centrifuged (10 min, 1539 g), and filtered. Distilled water (25 mL) was added to the filtrate, and the resulting mixture was shaken and again centrifuged (10 min, 1539 g). The organic phase was separated and dried by passing it through a small pad of anhydrous sodium sulphate. The solvent was removed in a rotary evaporator and then under a gentle stream of nitrogen to prevent lipid oxidation.

2.4. Determination of fatty acid by NMR

2.4.1. ^{1}H NMR conditions

A portion of each of the extracted lipid fractions (25 mg) was dissolved in 500 μL of $CDCl_3$ containing 0.03% TMS as internal reference and placed in standard 5 mm NMR tubes.

^{1}H NMR spectra were recorded in a 500 MHz Bruker Avance III spectrometer using a zg30 pulse sequence and spectral width of 10330.6 Hz. Acquisition was carried out at 298 K, collecting 65,536 data points after 24 scans, with a relaxation delay of 1 s and pulse width of 8.

2.4.2. Lipid quantification by ^{1}H NMR

All spectra were processed in the same way using the MestReNova software (Mestrelab Research, 2019). An exponential apodization of 0.3 MHz was applied to free induction decay data prior to Fourier transformation. Automatic phase correction using *Metabonomics* and *Regions Analysis* algorithms and *Splines* baseline correction gave best results and were routinely applied to all spectra. Next, the spectrum was referenced to the TMS signal, cut between 8 and −0.5 ppm and normalised to the highest peak. To ensure a homogeneous treatment of the spectra, all the

processing was systematically carried out using a process template. This allows for consistently processed spectra in minimal time.

^{1}H NMR signals were assigned according to previous studies (Nieva-Echevarría, Goicoechea, Manzanos María, & Guillén, 2015; Tsiafoulis et al., 2019) and integrated using MestReNova algorithm (Mestrelab Research, 2019). The values of the integrals allowed obtaining the percentages of triacylglycerides (TAG), 1,2-diacylglycerides (1,2-DAG), 1,3-diacylglycerides (1,3-DAG) and free fatty acids (FFA), and the different types of FA (ω-3, ω-6, polyunsaturated PUFA, monounsaturated MUFA, and saturated SFA), according to the equations described in Results and Discussion section.

2.5. Determination of fatty acids by gas chromatography

2.5.1. Transesterification

A portion of extracted fat from each sample (10 mg) was transesterified with methanol before being subjected to CG-FID analysis. Transesterification of FA was carried out according to the method described by Sandler and Karo (Sandler & Karo, 1992). Briefly, *n*-hexane (1 mL) containing tridecanoic acid (C13:0, 0.4 mg/mL) as an internal standard was added to the lipid extract sample (10 mg). Hexane was removed under a gentle stream of nitrogen and a 0.1 N solution of sodium methoxide (1 mL), prepared by dissolving metallic sodium in methanol, was incorporated. The mixture was vortexed and heated in an oven at 80 °C for 30 min. Then, 5% sulphuric acid in methanol (1 mL) was added, and the mixture was again vortexed and heated at 80 °C for further 30 min. Then, *n*-hexane (1 mL) and saturated aqueous NaCl (1 mL) were added, and the mixture was shaken and centrifuged at 3464 g 2 min. The supernatant phase containing fatty acid methyl esters (FAME) was withdrawn with a Pasteur pipette and placed into a 2 mL GC vial. The solvent was evaporated with a nitrogen flush and the residue was redissolved in *n*-hexane (1 mL).

2.5.2. Gas Chromatography analysis

FAME were analysed using a Hewlett–Packard HP-5890A gas chromatograph coupled to flame ionization detector (GC-FID). Cyanopropyl column (ZEBRON ZB 171 FAME, Phenomenex, California, USA; 20 m × 0.18 mm i.d. × 0.15 μm film thickness) with split injection (100:1) and helium at a constant flow of 2.7 mL/min as the carrier gas were used. Injector and detector temperatures were set at 250 °C. The temperature profile of the oven was 150 °C that increased at 10 °C/min to 180 °C; this was held for 1 min and increased again at 7 °C/min to 205 °C, which was maintained for 2 min.

FAME peaks were identified by comparison with suitable standards (Supelco 37-component FAME mix, Merck).

2.5.3. Fatty acid quantification by GC-FID

The external calibration curve method was applied for the quantification of FA. Five consecutive dilutions of the mix of standards (Supelco 37-component FAME mix, Merck) were prepared and the corresponding amounts of methyl tridecanoate (C13) standard (Merck) were added to keep the same concentration (0.4 mg/mL) of this FAME in the five dilutions. For each FAME, a calibration curve (FAME peak area/C13 peak area versus FAME amount/C13 amount) was constructed. The results, expressed in % mol, were calculated using the exact weight of the sample and the molecular weight of each FAME.

2.6. Statistical design

FA quantities obtained by GC-FID and NMR were compared by one-way analysis of variance (ANOVA). For this, the program IBM SPSS Statistics v.22 (IBM, 2013) was used.

Two multivariate data analysis methods (Principal component Analysis (PCA) (Bro & Smilde, 2014) and Decision Tree (DT) (Quinlan, 1987)) were used to assess the ability of NMR to classify the samples into different categories.

2.6.1. Principal component analysis

To check if it is possible to classify the hams according to their commercial category, the processed spectra between 8 and −0.5 ppm were divided into bins 0.1 ppm wide. The values of the integrals corresponding to each bin were normalised to the average of the 48 samples using the Reference Spectrum algorithm. The resulting data set was analysed using the SPSS package (IBM, 2013). The Principal Component Analysis (PCA) (Bro & Smilde, 2014) was performed after Friedman ranking (Friedman, 1940), so the most important bins of the NMR spectra were used in order to evaluate the distribution of the samples.

2.6.2. Decision tree (DT)

A DT was constructed from the bins corresponding to the areas of the spectra containing all the relevant peaks. Portions of the spectra showing only baseline noise or solvent peaks ($CDCl_3$, TMS) were not considered.

The DT classification task of data mining was performed by using the free software tool Waikato Environment for Knowledge Analysis (WEKA) (Eibe, Hall, & Witten, 2016). Specifically, the J48 DT algorithm (Quinlan, 1993) was applied according to the configuration described by Anyanwu and Shiva (2009) and Priyam, Abhijeeta, Rathee, and Srivastava (2013), considering a confidence factor of 0.5 and minimum bucket size of 3.

The statistical assessment of the classification performance was carried out by using the following classifications statistics (Demšar, 2006; Hand, 2012): sensitivity (SENS), specificity (SPEC), positive predictive value (PPV), negative predictive value (NPV), error rate, fall-out rate, false discovery rate (FDR), false omission rate (FOR), the critical success index (CSI), the accuracy and the F1 score. These parameters are given by the following equations:

$$SENS = \frac{TP}{TP + FN} \tag{1}$$

$$SPEC = \frac{TN}{FP + TN} \tag{2}$$

$$PPV = \frac{TP}{TP + FP} \tag{3}$$

$$NPV = \frac{TN}{TN + FN} \tag{4}$$

$$Error\ rate = \frac{FN}{TP + FN} \tag{5}$$

$$Fall\ out\ rate = \frac{FP}{FP + TN} \tag{6}$$

$$FDR = \frac{FP}{TP + FP} \tag{7}$$

$$FOR = \frac{FN}{TN + FN} \tag{8}$$

$$CSI = \frac{TP}{TP + FN + FP} \tag{9}$$

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN} \tag{10}$$

$$F1\ Score = \frac{2 \cdot TP}{2 \cdot TP + FP + FN} \tag{11}$$

where, TP and TN stand for True Positive and True Negative, respectively, accounting for the samples that have been correctly assigned as belonging (TP) or not belonging (TN) to a specific class. FP and FN stand for False Positive and False Negative, respectively, accounting for the samples that have been wrongly assigned as belonging (FP) or not

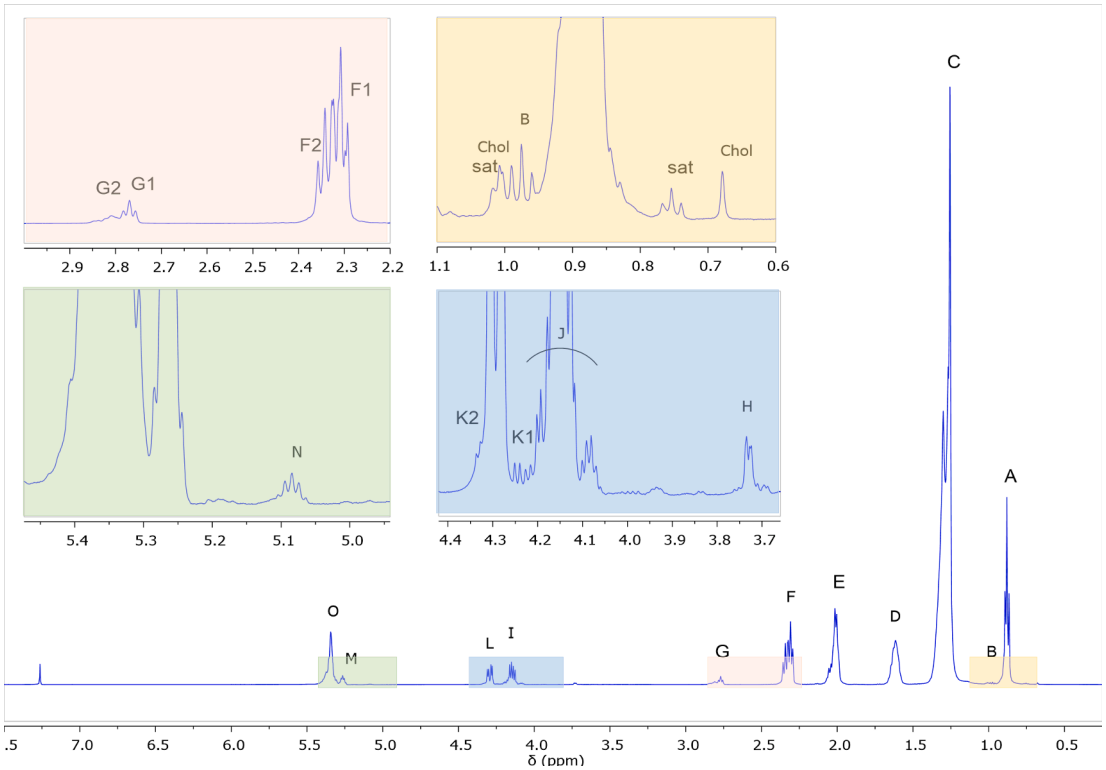

Fig. 1. ^{1}H NMR spectrum of a typical organic extract of Iberian ham. Inserts represent expansions of different regions, showing smaller peaks corresponding to minor components.

Table 1
^{1}H NMR peak assignment of a typical lipid extract from Iberian ham. Peak labels (**A** to **O**) agree with those given in Fig. 1.

Peak	δ (ppm)	Multiplicity, *J* (Hz)	Assignment
Chol	0.68	s	CH_3, cholesterol C-18
sat		t	^{13}C satellites of peak **A**
A	0.88	t, *J* = 6.9 Hz	terminal CH_3 (all FA except ω-3)
B	0.98	t, *J* = 7.4 Hz	terminal CH_3 (ω-3 FA)
C	1.41–1.19	m	$(CH_2)_n$ (all FA)
D	1.69–1.55	m	β-CH_2 (all FA except EPA and ARA)
E	2.11–1.94	m	=CH-CH_2 (MUFA and PUFA)
F1	2.33–2.27	m	α-CH_2 (all FA in TGA)
F2	2.39–2.32	m	α-CH_2 (all FFA)
G1	2.77	t, *J* = 6.7 Hz	=CH-CH_2-CH= (PUFA, diunsaturated)
G2	2.89–2.79	m	=CH-CH_2-CH= (PUFA polyunsaturated)
H	3.74	dd, *J* = 5.0, 1.8 Hz	CH_2OH (glyceryl 1,2-DAG)
I	4.15	dd, *J* = 11.9, 6.0 Hz	CH_2 (glyceryl TAG)
J	4.22–4.06	m	CH_2OH and CHOH (glyceryl 1,3-DAG)
K1	4.25	dd, *J* = 11.9, 5.5 Hz	CH_2OR (glyceryl 1,2-DAG)
L	4.29	dd, *J* = 11.8, 4.3 Hz	CH_2 (glyceryl TAG)
M	5.29–5.23	m	CH (glyceryl TAG)
K2	c.a. 4.33	dd overlaped	CH_2OR (glyceryl 1,2-DAG)
N	5.08	p, *J* = 5.1 Hz	CHOR (glyceryl 1,2-DAG)
O	5.43–5.29	m	CH = CH (MUFA and PUFA)

belonging (FN) to a specific class.

3. Results and Discussion

3.1. NMR peak assignment and quantification

The ^{1}H NMR spectrum of a representative Iberian ham lipid extract, depicted in Fig. 1, shows all the characteristic signals of glycerides and FA present in ham. An alphanumeric nomenclature is used to identify each signal, following a descending field order. Assignment of the different signals is based on previous reports on the ^{1}H NMR analysis of fats (Nieva-Echevarría et al., 2015; Tsiafoulis et al., 2019) and is summarized in Table 1. Thus, the peaks between 0.88 and 2.79 ppm (peaks **A** to **G**) correspond to aliphatic protons bonded to sp^3-hybridized carbons of FA chains, both from glycerides and FFA. Particularly, signal **G** corresponds to bis-allylic protons of polyunsaturated fatty acids (PUFA), and its shape depends on the proportion of ω-3 and ω-6 PUFA. A signal corresponding to the protons on sp^2-hybridized carbons of unsaturated FA can also be observed between 5.29 and 5.43 ppm (peak **O**). Peaks **I**, **L** and **M** correspond, respectively, to CH_2 and CH groups of the glyceride backbone of TAG. The occurrence of smaller peaks **H**, **J**, **K** and **N** evidences the presence of products of the partial hydrolysis of TAG. As peaks **H** and **N**, respectively due to CH_2 and CH of 1,2-DAG, are relatively isolated and well resolved, it is possible to discriminate between 1,2- and 1,3-DAG isomers.

Thus, a simple visual inspection of the ^{1}H NMR spectrum allows to roughly determine the proportion of SFA, MUFA and PUFA fatty acids, and also the extent of TAG hydrolysis.

Importantly, the integrals of the ^{1}H NMR peaks are proportional to the number of protons contributing to these peaks. Thus, identification of singular peaks corresponding to a particular type of compound makes it possible to quantify this compound in relation to other components. Although some peaks correspond to more than one type of compound and other peaks are overlapped or not well resolved, a number of peaks characteristic of particular types of compounds are sufficiently well resolved to calculate the corresponding integrals. Accordingly, equations can be deduced to calculate the proportion of different types of FA and the products of hydrolysis of TAG.

Peak **F**, due to methylene protons α to the carboxyl group of all FA, was used to normalize the integrals of the rest of the peaks in the spectrum. Some authors have used the glycerol signal for this purpose, mainly in vegetal oils, but in this case, this was not possible due to the presence of FFA and DAG. Some peaks that are small or partially overlapped with contiguous signals could be optimally integrated using the

Table 2
Mean values of the molar percentages of FA in dry-cured Iberian ham samples from different labels and commercial brands, obtained by GC-FID and NMR methods, and the p-values resulting from the statistical treatment comparing both methods.

	ω-3			ω-6			PUFA			MUFA			SFA		
sample	*GC*	*NMR*	*P*	*GC*	*NMR*	*P*	*GC*	*NMR*	*P*	*GC*	*NMR*	*P*	*GC*	*NMR*	*P*
Black															
1	0.59 ± 0.06	0.64 ± 0.10	0.70	7.74 ± 0.38	8.07 ± 0.96	1.00	8.33 ± 0.36	8.71 ± 0.87	0.70	55.72 ± 0.90	54.51 ± 1.43	0.70	35.95 ± 0.64	36.78 ± 1.59	1.00
2	0.46 ± 0.01	0.62 ± 0.18	0.10	8.82 ± 0.44	9.27 ± 1.83	0.70	9.28 ± 0.44	9.89 ± 1.91	0.70	53.91 ± 0.61	52.73 ± 0.93	0.20	36.82 ± 0.62	37.38 ± 1.79	1.00
3	0.38 ± 0.02	0.53 ± 0.20	0.70	6.64 ± 0.24	5.53 ± 0.91	0.20	7.02 ± 0.26	6.05 ± 0.98	0.40	57.07 ± 0.79	56.63 ± 0.79	0.70	35.91 ± 0.68	37.36 ± 1.27	0.20
4	0.46 ± 0.01	0.64 ± 0.06	0.10	7.90 ± 0.79	7.79 ± 1.62	1.00	8.36 ± 0.79	8.43 ± 1.68	1.00	58.59 ± 2.46	58.96 ± 3.12	0.70	33.05 ± 1.67	32.61 ± 1.55	0.70
Red															
5	0.64 ± 0.04	0.74 ± 0.25	0.70	9.16 ± 0.92	8.58 ± 1.89	1.00	9.79 ± 0.90	9.32 ± 2.05	1.00	55.30 ± 0.57	54.31 ± 0.57	0.20	34.91 ± 0.36	36.38 ± 2.56	0.70
6	0.84 ± 0.05	0.92 ± 0.24	0.70	8.71 ± 0.09	8.34 ± 1.82	0.70	9.55 ± 0.14	9.26 ± 2.05	0.70	54.67 ± 0.97	52.39 ± 2.05	0.20	35.79 ± 0.91	38.35 ± 4.10	0.70
7	0.42 ± 0.04	0.48 ± 0.16	0.70	6.78 ± 0.07	5.80 ± 0.69	0.10	7.20 ± 0.04	6.27 ± 0.82	0.20	57.82 ± 0.70	57.29 ± 0.99	1.00	34.98 ± 0.73	36.43 ± 0.35	0.10
8	0.64 ± 0.04	0.90 ± 0.23	0.20	7.65 ± 0.56	7.62 ± 1.27	0.70	8.29 ± 0.59	8.52 ± 1.48	0.70	54.39 ± 0.18	53.15 ± 0.19	0.10	37.32 ± 0.76	38.33 ± 1.65	0.40
Green															
9	0.59 ± 0.02	0.67 ± 0.34	0.70	6.34 ± 0.12	6.43 ± 0.19	1.00	6.94 ± 0.11	7.10 ± 0.39	0.70	53.58 ± 1.13	52.27 ± 1.35	0.40	39.48 ± 1.09	40.63 ± 1.28	0.40
10	0.33 ± 0.04	0.52 ± 0.07	1.00	6.45 ± 0.20	6.01 ± 0.43	0.20	6.78 ± 0.21	6.52 ± 0.39	0.70	51.57 ± 1.50	51.17 ± 1.41	0.40	41.65 ± 1.30	42.31 ± 1.53	0.40
11	0.48 ± 0.02	0.62 ± 0.14	0.40	8.46 ± 0.35	8.60 ± 1.01	0.70	8.93 ± 0.33	9.22 ± 1.14	0.70	53.40 ± 0.79	51.26 ± 1.26	0.10	37.67 ± 1.12	39.52 ± 1.12	0.20
12	0.30 ± 0.05	0.45 ± 0.10	0.20	6.37 ± 0.02	6.21 ± 0.56	0.70	6.68 ± 0.06	6.66 ± 0.54	1.00	54.74 ± 0.58	53.86 ± 1.85	1.00	39.14 ± 0.63	39.48 ± 1.36	1.00
White															
13	0.45 ± 0.17	0.53 ± 0.02	0.40	7.22 ± 0.77	6.44 ± 0.63	0.70	7.67 ± 0.77	6.97 ± 0.73	0.40	52.66 ± 1.16	51.52 ± 2.09	0.40	39.67 ± 1.46	41.51 ± 2.82	0.40
14	0.37 ± 0.04	0.39 ± 0.05	1.00	7.26 ± 0.31	5.78 ± 1.72	0.70	7.64 ± 0.30	6.17 ± 1.74	0.70	50.14 ± 1.57	54.85 ± 7.02	0.70	42.23 ± 1.85	38.98 ± 5.44	0.70
15	0.43 ± 0.04	0.72 ± 0.09	0.10	7.08 ± 0.14	8.39 ± 0.73	0.10	7.51 ± 0.17	9.11 ± 0.78	0.10	51.34 ± 0.26	51.12 ± 0.92	0.70	41.15 ± 0.31	39.77 ± 1.68	0.70
16	0.38 ± 0.01	0.39 ± 0.07	1.00	6.29 ± 0.10	6.04 ± 0.14	0.10	6.67 ± 0.09	6.43 ± 0.09	0.10	54.56 ± 0.32	53.13 ± 1.41	0.20	38.78 ± 0.28	40.44 ± 1.44	0.10

MestreNova *peak* integration method (Mestrelab Research, 2019), which applies a deconvolution algorithm prior to integration. However, most larger and well resolved peaks did not require deconvolution and better results were obtained using the conventional *sum* integration method (Mestrelab Research, 2019). To minimize possible errors, integration intervals had to be carefully delimited. Furthermore, in order to apply consistent conditions to all samples, the processing of the ^{1}H NMR spectra, including integration, was automated using appropriate processing templates and scripts.

3.2. Fatty acid quantification in Iberian hams

The proportion of ω-3 FA can be directly calculated from the integral value of peak **B** divided by three, as it corresponds to the three protons of the terminal methyl group of this type of FA. The integral value of peak **G** accounts for the bis-allylic protons of all PUFA and, in a good approximation, this is two protons from di-unsaturated ω-6 FA and four protons from tri-unsaturated ω-3 FA. Accordingly, ω-6 FA can be calculated as (**G**/2) – (**B**/3). Obviously, the amount of PUFA is the sum of ω-3 and ω-6 FA. Peak **E** corresponds to allylic methylene groups in PUFA and MUFA. As the number of allylic protons is always four, the proportion of MUFA can be readily calculated as (**E**/4) – (**G**/2) + (**B**/3). After normalizing all these values, the rest to complete 100% accounts for the percentage of SFA. These equations are summarised below (equations (12) to (16)).

$$omega3 = B/3 \tag{12}$$

$$omega6 = (G/2) - (2B/3) \tag{13}$$

$$PUFA = omega3 + omega6 \tag{14}$$

$$MUFA = (E/4) - (G/2) + (B/3) \tag{15}$$

$$SFA = 100 - MUFA - PUFA \tag{16}$$

To validate the NMR quantification, all samples were parallelly analysed by GC-FID. The results of this type of analysis are usually expressed in mg of FA per 100 g of sample. However, in this study, the FA mass values obtained by GC were divided by each individual molecular weight to transform them into molar percentage, and then added by type (ω-3, ω-6, MUFA and SFA) to compare them with the data obtained from the ^{1}H NMR analysis. The means of the results of each commercial brand of dry-cured Iberian hams, obtained by both GC-FID and ^{1}H NMR, and their respective deviations are summarized in Table 2, together with the *p*-values that compare these methods.

Successfully, although the deviations obtained by NMR analysis were somewhat greater than those obtained by GC, there were no significant differences between both methods, since all p-values were >0.05.

Regardless of the type of analysis performed, our data show that MUFA predominate over other types of FA, ranging from 51% to 59%. In addition, its proportion increases significantly in parallel with the quality of the ham, being higher in Black and Red Label hams than in White and Green Label hams. These results are in agreement with previous studies by GC analysis that found consistently higher contents of MUFA in the meat of pigs fed on acorns (Pérez Palacios et al., 2009;

Table 3
Mean values of the molar percentages of FA forming part of TAG, 1,2-DAG, 1,3-DAG and FFA obtained by ^{1}H NMR for each category in samples of cured Iberian ham of different commercial brands and the p-values resulting from the statistical treatment comparing categories.

LABEL (category)	TAG	1,2-DAG	1,3-DAG	FFA
Black	76.42 ± 2.38	1.90 ± 0.36	4.08 ± 1.03	17.59 ± 1.55
Red	76.94 ± 7.67	1.95 ± 0.5	4.10 ± 1.54	17.01 ± 5.82
Green	72.29 ± 7.25	1.85 ± 0.28	3.83 ± 1.00	22.03 ± 6.36
White	67.00 ± 12.30	2.46 ± 0.54	5.66 ± 1.78	23.79 ± 10.26
P	0.12	0.02	0.03	0.30

Tejeda, Gandemer, Antequera, Viau, & García, 2002).

A similar trend was observed in the results for PUFA, which nevertheless showed markedly lower values, between 6% and 9%. On the contrary, the values obtained for SFA ranged between 42% and 32%, being lower in the higher quality hams. These differences in the FA profile of hams from pigs subjected to different diets are attributed to the high content of unsaturated fatty acids (principally oleic acid) in acorn, the main dietary contribution of pigs in montanera (Pérez Palacios et al., 2009; Tejeda, Gandemer, Antequera, Viau, & García, 2002).

3.3. Quantification of triglyceride hydrolysis products in Iberian hams

Decisively, the concentration of DAG and FFA increases during the curing process due to lipolysis. This begins with the acidic and enzymatic hydrolysis of TAG leading to 1,2-DAG. A slower extended transformation isomerizes 1,2-DAG to thermodynamically more stable 1,3-DAG and, finally, DAG are hydrolysed to monoacylglycerides (MAG) and FFA (Narváez-Rivas, Vicario, Constante, & León-Camacho, 2007).

The profile of TAG's hydrolysis products reflects both the quality of the raw material and the type and extent of the ripening process, and thus can be used as a quality control parameter.

The ^{1}H NMR signals corresponding to the glycerol framework occur in the region of 3.7–5.1 ppm. There are two well resolved peaks from 1,2-DAG. Peak **H** corresponds to the glycerol methylene group attached to a free OH in 1,2-DAG. Peak **N** corresponds to the middle CH glycerol group in 1,2-DAG. The resolution of peak **H** is usually better, so we have used it to calculate the percentage of 1,2-DAG. As there are two fatty acid chains in DAG, the proportion of FA that are part of 1,2-DAG is 2 **N** or 2(**H**/2) = **H**. On the other hand, signal **K2** corresponds to the protons of the glycerol methylene attached to an O-acyl group in 1,2-DAG. This signal overlaps with peak **L**, corresponding to two glycerol methylene protons of TAG. Thus, the proportion of fatty acids that form part of triglycerides can be calculated as 2/3 (**L-H**). Peak **I** also corresponds to two glycerol methylene protons of TAG, and overlaps with signal **J** from the five glycerol protons of 1,3-DAG. Thus, knowing the value of TAG, it is possible to calculate 1,3-DAG using the equation 2/5(**J**+**H-L**). The fatty acids in TAG, DAG, MAG and phospholipids (PL), together with FFA, must add up to a total of 100%, so the difference between 100 and the sum of TAG and DAG corresponds to MAG, FFA and PL, being FFA the main component of the three. The amount of MAG and phospholipids in all the analysed samples can be considered negligible, since no apparent signals corresponding to these compounds are evident in any of the ^{1}H NMR spectra. The equations to calculate the proportions of fatty acids, either in its free form or being part of the different acylglycerides are summarised below.

$$TAG = 2/3(L - H) \tag{17}$$

$$1,2DAG = H \tag{18}$$

$$1,3DAG = 2/5(J + H - L) \tag{19}$$

$$FFA = 100 - 1,2DAG - TAG - 1,3DAG \tag{20}$$

The means and deviations of the percentages of TAG and their hydrolysis products obtained by applying the equations to the ^{1}H NMR spectral data of three samples of each commercial brand and category are summarized in Table 3.

Although these values present a fairly large dispersion, this is not surprising, as large differences are likely to occur in the curing process conditions, such as time, temperature and humidity, applied by different producers (Fernandes et al., 2014). However, even though the degree of

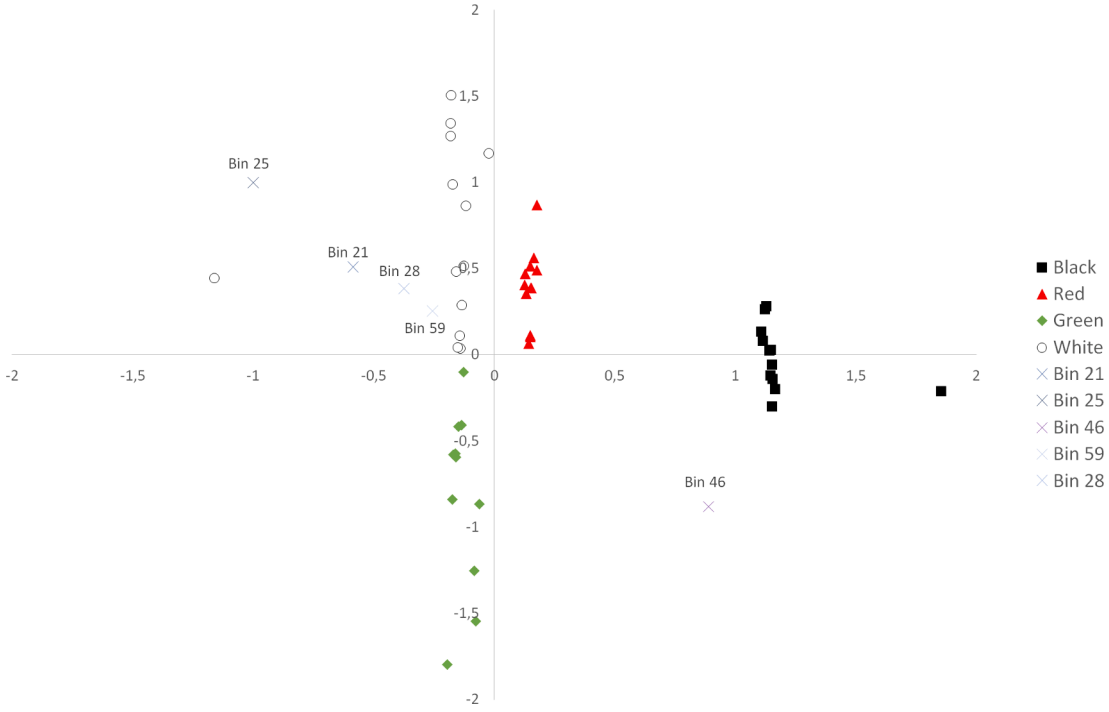

Fig. 2. Principal Component Analysis: discriminant analysis prediction of the 48 Iberian ham subsamples. Samples cluster according to their commercial category. The image also shows the five most important bins resulting from the Friedman ranking.

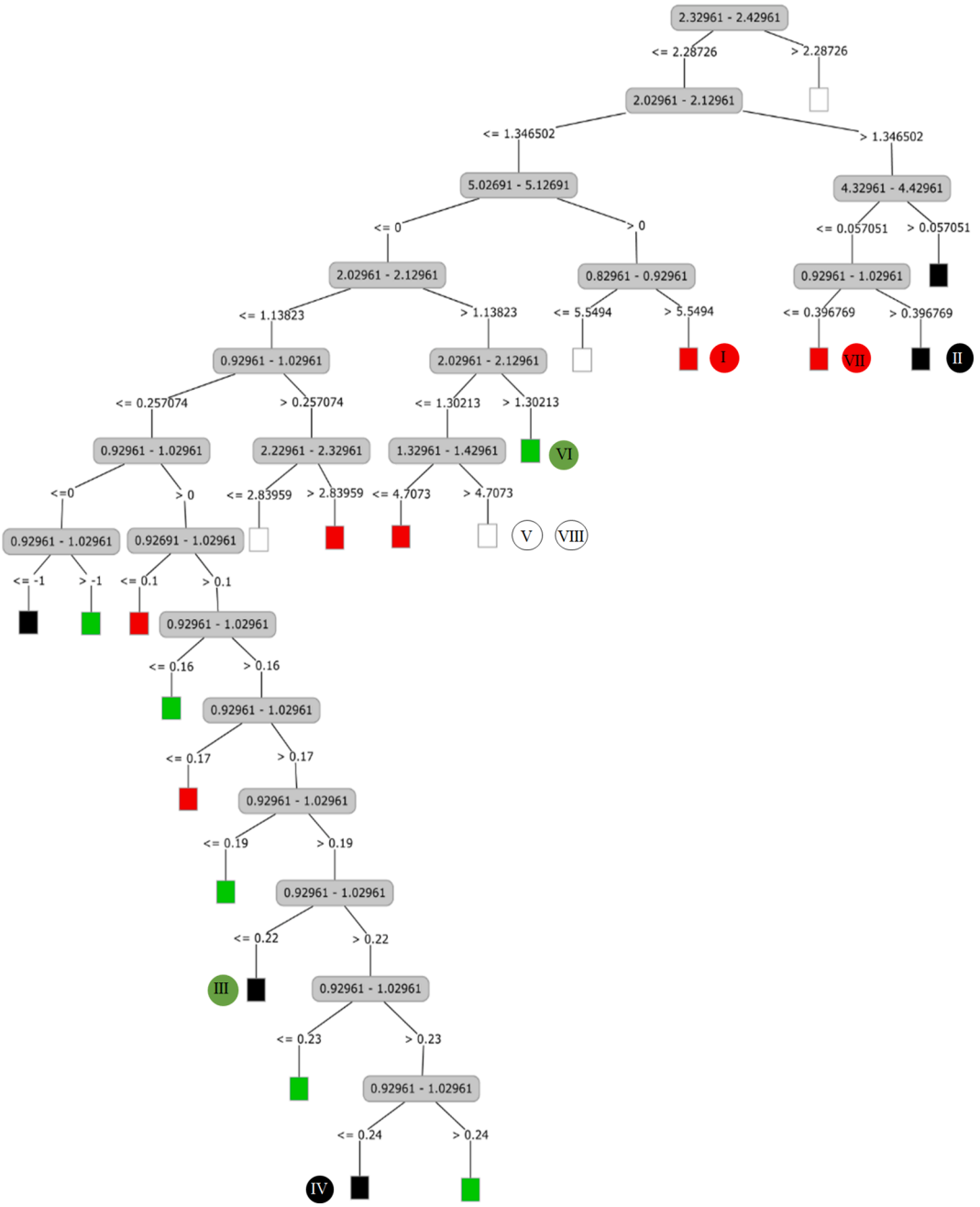

Fig. 3. Decision tree: discriminating model that classifies original Iberian ham samples, represented as squares (each square account for one or more samples) according to their category (labels), and classification of problem samples, represented as circles.

hydrolysis can be largely attributed to differences in the curing process, there is an apparent decreasing trend in the percentage of DAG from lower quality hams to higher quality hams. Greater dispersions are also observed in hams from pigs reared on concentrate feeds (White label), since each farmer commonly use concentrate feeds of different composition, while pigs reared in montanera receive a more homogeneous diet.

GC-FID cannot provide information on the extent of lipolysis, as these procedures involve the derivatization all acylglycerides and FFA into fatty acid esters. Complex and time-consuming techniques are generally required to separate and quantify the different products of lipolysis. Even more complex methods, based on MS are not able to discriminate 1,2-DAG from isomeric 1,3-DAG (Fernandes et al., 2014). Advantageously, NMR spectroscopy of the raw lipid extract makes it possible to visualize and quantify TAG's hydrolysis products simultaneously with the fatty acid profile.

The trend in the values obtained from the NMR spectra are in general agreement with previously published studies based on pigs classified according to their feeding regime as "Cebo" (concentrate feed), "Recebo" (concentrate feed and acorns) and "Bellota" (acorns), using ionization mass spectrometry after thermal imprinting (Fernandes et al., 2014) and column fractionation followed by GC analysis (Narváez-Rivas et al., 2007).

3.4. Chemometric approach for classification and authentication of Iberian hams

Principal component analysis (PCA) was applied to ^{1}H NMR data to evaluate the capability of this technique as a tool to classify dry-cured Iberian ham categories. A Friedman ranking (Friedman, 1940) was carried out in order to obtain the 20 bins of the NMR spectra that

contribute more significantly to discriminate the four groups of samples. Then, a PCA was carried out from these 20 most significant bins. The first two principal components accounted for 90.89% of the total variance (74.68% for PC1, and 16.21% for PC2), which is well above the recommended minimum 65% (Bro & Smilde, 2014).

The two-dimensional scores plot representing these two first principal components (Fig. 2) shows the samples clearly clustering into four well-differentiated groups, each corresponding to a different ham category. White label hams group in the first quadrant, with negative scores on PC1 and positive scores on PC2. Green label hams cluster in the second quadrant, characterised by both negative scores on PC1 and PC2. Red label hams cluster in the third quadrant, having positive scores on PC1 and PC2. Finally, Black label hams have significantly more positive scores on PC1 than Green labels and group at both positive and negative PC2 values.

Variables contributing to PC1 and PC2 with highest loading values are bins corresponding to interval 1.929–2.029 ppm (bin 25; allylic methylene groups in MUFA and PUFA), interval 4.029–4.129 ppm (bin 46; 1,3-DAG) and the interval 5.229–5.329 ppm (bin 59; unsaturated FA). Accordingly, clustering of the different ham commercial categories can be attributed mainly to the unsaturation of fatty acids, i.e., the amount of PUFA and MUFA, as well as the abundance of 1,3-DAG. The two remaining bins, 1.529–1.629 (bin 21) and 2.229–2.329 and (bin 28), correspond respectively to protons in α and β to the FA carbonyl carbon. As they are present in all FA, they can serve as a reference for the normalisation of all the ham samples. These variables include both animal feeding factors and product processing factors. The degree of unsaturation of fatty acids has often been used for the classification of foods. Thus, the ^{1}H NMR signals corresponding to allylic and vinylic protons have shown to be discriminating peaks with respect to animal feeding. For example, they have been used to discriminate between organic and conventional bovine milk (Tsiafoulis et al., 2019) and between wild and farmed sea bass (Mannina et al., 2008). On the other hand, the signals corresponding to DAG are indicators of the degree of TAG hydrolysis and have thus revealed as important variables related to cured meat maturation process (Narváez-Rivas et al., 2007).

In addition to PCA, a decision tree (DT) was carried out from the set of 48 initial lipid samples. Fig. 3 shows the resulting DT and the intervals that discriminate the different ham categories.

The statistical results obtained are: SENS $>$ 0.73, SPEC $>$ 0.92, PPV $>$ 0.79, NPV $>$ 0.92, error rate $<$ 0.16, fall-out rate $<$ 0.08, FDR $<$ 0.21, FOR $<$ 0.08, CSI $>$ 0.62, accuracy $>$ 0.90 and F1 score $=$ 0.76. These indicate a very good classification according to Yerushalmy (1947). Therefore, the decision tree makes a correct sorting of the samples, leaving out 8 of the 48 initial subsamples. An improved classification would surely be achieved by increasing the number of samples.

Determining intervals in the classification correspond to DAG (4.33–4.43 and 5.03–5.13) and FA unsaturation (2.03–2.13). The discriminating capacity of these two factors was already shown in the PCA analysis. Other relevant bins directly or indirectly related to unsaturation are 0.93–1.03 and 0.83–0.93, corresponding to the terminal methyl groups in respectively non ω-3 and ω-3 FA, and 1.33–1.43, which correspond to protons on unsaturated carbons. On the other hand, the intervals 2.23–2.33 and 2.33–2.43 correspond to the protons on FA alpha carbons, which particular resonance frequency is shifted to lower fields in hydrolysed products (DAG and FFA) with respect to TAG (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014).

To verify the classification capacity of this decision tree, test Iberian ham samples purchased from different commercial establishments were subjected to the same discrimination routine. A total of eight ham samples from diverse categories and commercial brands were subjected to the DT routine. These test samples underwent the same lipid extraction and NMR processing as the initial set of samples, and the resulting spectra were as well normalised against the spectra of the original set of samples. Fig. 3 shows the intervals that were found to discriminate the different categories of hams in the DT. The original samples are represented as squares, while the 8 problem samples are represented as circles. Successfully, 7 in 8 samples were correctly classified in their respective groups and only one was wrongly classified as "Green label" when it was actually "Black label", which represents a success rate of 87.5%.

4. Conclusion

High resolution ^{1}H NMR spectroscopy has proven to be a powerful tool for the lipidomic analysis of Iberian hams. This methodology has shown to be a good alternative or a complementary approach to other stablished analytical methods, such as GC-FID, as it requires minimal sample handling and provides reliable quantitative and qualitative information. The lipidic extract was analysed directly, with no need for derivatization, providing the actual picture of the metabolites present in the sample. The proportion of SFA, MUFA, ω-6 FA and ω-3 FA was readily calculated from the integrals of the peaks. Furthermore, ^{1}H NMR spectroscopy allowed to obtain the proportion of TAG and their different hydrolysis products, such as 1,2-DAG, 1,3-DAG and FFA. ^{1}H NMR combined with simple chemometric analysis, such as PCA or DT, allowed to classify Iberian hams in the four categories stablished by Spanish regulations.

In future studies, the possibility of using smaller and more affordable low resolution NMR spectrometers will be addressed. This would surely facilitate the possibility of performing quality control quickly and economically in companies dedicated to the commercialization of cured meat products. As well as a complete analysis of the spectrum, studying minority compounds present, since they are important in the classification method exposed.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Abraham Pajuelo reports financial support was provided by Extremadura Employment Public Service (TE-0056-19).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132371.

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