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RESEARCH ARTICLE

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Discriminant analysis using fatty acids profile, stable carbon isotopes and tocopherols content as tool for feeding system prediction in Iberian pigs

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Abstract

Aim of study: The application of three analytical methods (fatty acids: FA, tocopherols: TOC, and isotope ratio: ISO) to distinguish the feeding type received by Iberian pigs during the fattening stage.

Area of study: This distinction is very important for the labelling of Iberian high-quality products in the *Quercus* forest located on the southwest of Iberian Peninsula, where several production systems coexist.

Material and methods: Discriminant analysis on fat samples with unknown background obtained from commercial pigs was applied. The feasibility of the combination method to determine the authentication of feeding background was studied on samples from different fattening system: free-range feeding with acorn and pastures (BE); free-range feeding acorn and pastures plus commercial feeds (RE); open-air feeding with commercial feeds (CA); standard feeding with commercial feeds (CE).

Main results: In a first application of the methods, the overall success rate was 60.1% for FA, 49.7% for ISO and 49.3% for TOC. When some of the batches were reclassified attending to those previous results and additional information available about farm characteristics, ISO and TOC analyses had a 70% of success rate in the four categories, whereas FA showed 40.5%, attributable to the use of high-oleic commercial diets. The predictions improved with the method combination. The ISO+TOC combination achieved a 84.1% of success in prediction. When it was reduced to just two categories (acorn vs non-acorn), the success reached a 95% for FA+TOC and ISO+TOC.

Research highlights: The use of these methods as a complementary tool for quality controls is highly recommended to avoid undesirable misclassifications.

Additional key words: traceability; analytical methods; feeding background; autochthonous breed.

Abbreviations used: BE (free-range feeding with *ad libitum* intake of acorn and pastures); CA (open-air system feeding with commercial feeds besides of the grazing of grass or stubble); CE (standard feeding with commercial diet); FA (fatty acids profile); ISO (isotope analysis); RE (free-range feeding with *ad libitum* intake of acorn and pastures plus commercial feed); S1-S4 (season with data 1 to 4); TOC (tocopherols content).

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Introduction

Iberian pig production is aimed towards obtaining cured products (hams, shoulders and loins) of high organoleptic quality. In this context, several production systems coexist in the southwest Iberian Peninsula, from the one named *Montanera*, in which the pigs take advantages of natural resources (mainly acorns and pastures)

during the final fattening period (López-Bote, 1998), to the intensive one with in-door management completely based on commercial feeds. In the first one, the pigs are raised with a strong restriction of feeding until 100 kg approximately, with 12-14 months of age, and then they consume acorn and pasture until 150-160 kg of final weight, with 15-17 months of age. In the second, the pigs (most of them crossed with Duroc), grow and fatten close to *ad libitum* feeding, up to 150-160 kg reaching 10 months of age. Between these two extreme systems, there are other based on the combination of natural resources with different use of the territory and commercial mixed diets.

This productive diversity have made necessary a legislative effort (BOE, 2001, 2007 and 2014) to clarify the labelling of products through a mandatory implementation of a full traceability system (from the farm to the table) in order to protect consumers against fraudulent practices. The quality of the final product, and therefore its price, is directly linked to the production system, being more expensive the products from pigs with acorn consumption, of higher organoleptic qualities. This is because the feeding time with natural resources in free-range conditions has been directly associated with an accumulation of monounsaturated fatty acids, antioxidants and other compounds in pig tissues (Rey *et al.*, 2006) that confers a particular taste and flavour. However, free-range system (*Montanera*) is limited since it depends on the acorn and grass availability; meanwhile the intensive production is widespread being more accessible to the consumer due to its lower price.

In the last 25 years, several research groups have dealt with the development of appropriate analytical methods to differentiate the feeding background of Iberian pigs at the final fattening period. Many of them were based on the quantification of different compounds present in the diets, such as the determination of fatty acids profile (Ordóñez *et al.*, 1996), alpha- and gamma-tocopherols (Rey *et al.*, 2006), triglycerides (Viera-Alcaide *et al.*, 2007), neophytadiene (Tejeda *et al.*, 1999), or carbon stable isotope ratio (Recio, 2010; Delgado-Chavero *et al.*, 2013); and others based in the use of equipment such as near infrared spectroscopy (NIR) (García-Olmo *et al.*, 2009), chemsensor technique (Carrasco & Duque, 2013) and ionic mobility (Alonso *et al.*, 2008). Recently, González Domínguez *et al.* (2020) have proposed a new method to classify cured hams based on linear discriminant analysis and artificial neural network applied to fatty acid profile. However, only the method based in the determination of the fatty acids profile was applied for several years in the official standard contracts of pig sales. Later, it was demonstrated that some of these methods had a high level of success in *blind* tests of subcutaneous fat samples (without prior knowledge of their origin before the analysis and prediction) from animals with safely and reliably feeding systems (García-Casco *et al.*, 2013). Hence, Delgado-Chavero *et al.* (2013) reported a success rate of 85% on a total of 734 samples when using a discriminant analysis that combined the fatty acid profile and the $^{13}C/^{12}C$ isotope ratio of the four major fatty acids (palmitic, stearic, oleic and linoleic). Moreover, Rey *et al.* (2013) obtained in the same samples a 76% of accuracy using α-and γ-tocopherols as differentiating factors and a success rate of 80% when samples were dry-cured (Rey *et al.*, 2014). However, this methodology was not tested in combination with others such as the fatty acid profile or the $^{13}C/^{12}C$ isotope ratio. Moreover, there is not much information of the effect of method combination for pig feeding differentiation.

The aim of this study was to apply three individual methods (fatty acids, tocopherols and isotope ratio analyses) on fat samples taken from commercial pigs in which the feeding background was unknown, using a discriminant model previously built with a reliable database generated during three seasons. The second purpose was to further improve the discrimination of the feeding background by the combination of different methods. The overall objective is to provide a general and independent statistical system of prediction with ability to engage the most accurate methods of previous studies.

Material and methods

Samples collection

Samples of subcutaneous fat were taken near the rump area during three seasons, from December to March (season 2008/09, S1; 2009/10, S2; 2010/11, S3) from pigs raised under controlled feeding which corresponded to the four traditional production systems: free-range *Montanera* system with *ad libitum* intake of acorn and pastures (named *bellota*, BE); free-range system similar to the previous one but with some intake of commercial feed besides acorn and pastures (*recebo*, RE); mixed system where an open-air final period of at least 60 days is mandatory and the pigs are mainly fattened with commercial feeds, but they can take advantage of the grazing of seasonal grass or stubble until reach 150-160 kg and 12 months of age (*campo*, CA); and pigs fattened under the intensive system previously explained based on standard commercial diet (*cebo*, CE). Table 1 shows the number of controlled batches (group of pigs fattened on the same farm, with the same feeding system and slaughtered the same day in the same slaughterhouse) and animals according to season and feeding system.

During the fourth season (2011/12, S4) the samples were collected from 24 batches of Iberian pigs and their classification according to the type of feeding was assumed to be the category issued by Protected Denominations of Origin and by control organisations following

Table 1. Total number of samples and batches (between brackets) analysed over the four seasons (S1-S4) and batch code of the fourth season (S4)

	S1	S2	S3	S4	S4 Code
BE	57 (2)	25 (1)	135 (10)	135 (11)	BE1-B11
RE	40 (2)	77 (3)	72 (4)	49 (4)	RE1-RE4
CA	72 (3)	74 (3)	47 (3)	62 (5)	CA1-CA5
CE	31 (1)	24 (1)	80 (5)	50 (4)	CE1-CE4
Total	200 (8)	200 (8)	334 (22)	296 (24)	

BE: free-range with *ad libitum* intake of acorn and pastures. RE: free-range with *ad libitum* acorn and pastures plus commercial feed. CA: open-air system feeding with commercial feeds besides of the grazing of grass or stubble. CE: standard feeding with commercial diet.

the Regulation (EC) 765/2008 and the Standard UNE EN ISO/IEC 17020 (Table 1). Therefore, in the first three seasons the type of feeding was well known and reliable, while the fourth season was considered as the problem one, with some knowledge of the feeding system in some batches but no information at all in others.

Laboratory analysis

The samples of subcutaneous fat were analysed following three analytical methods: fatty acid profile (FA) (Ordóñez *et al.*, 1996), alpha- and gamma-tocopherols determination (TOC) (Rey *et al.*, 2006) and $^{13}C/^{12}C$ isotope ratio of the fatty acids methyl esters (ISO) (Recio *et al.*, 2013; Delgado-Chavero *et al.*, 2013).

Fatty acid analysis

Fatty acid analysis was carried out following the method described by Delgado-Chavero *et al.* (2013). Methyl esters were prepared by the addition of 0.2 mL of 2 M KOH in methanol and 4 mL of methanol to 0.2 g of total lipids, obtained by fusion in a microwave, and final centrifugation after 30 minutes. Gas chromatography (GC-FID) was carried out on two Perkin Elmer chromatographs (Waltham, Massachusetts, USA) with autosamplers and a fused silica capillary column (30 m × 0.32 mm internal diameter and 0.25 µm film thickness). The injector temperature was kept at 230°C and the detector temperature was 250 °C, with helium as the carrier gas. The percentage of 12 fatty acids (C12:0, C14:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C20:0 and C20:1) were obtained in these conditions.

Tocopherol quantification

Concentrations of alpha- and gamma-tocopherols in subcutaneous fat were quantified as described in Rey *et al.* (2006). Samples were saponified in the presence of KCl and KOH. Tocopherols were extracted with hexane and the upper layer was evaporated and dissolved in ethanol prior to analysis by reverse phase HPLC (HP 1100, provided with a diode array and fluorescence detector) (Agilent Technologies, Waldbronn, Germany). Separation was carried out on an Agilent Technologies Lichrospher RP-C18 column (250 mm × 4 mm i.d., 5 µm particle size), the mobile phase was methanol/water (97:3 v/v) at a flow rate of 2 mL/min and peaks were recorded at 292 nm. Peaks were detected by a fluorescence detector (Agilent technologies series 1200) set at λ-excitation: 295 nm and λ-emission: 330 nm. Analyses were carried out in duplicate. The identification and quantification of both tocopherols was carried out by means of a standard curve (R^2=0.9999) developed with the pure compounds (Sigma, Alcobendas, Madrid). Results were expressed as µg of gamma-tocopherol or alpha-tocopherol per gram of fresh matter.

Analysis of the fatty acids methyl esters (FAMEs) isotopes

The $^{13}C/^{12}C$ isotope ratio ($\delta^{13}C$) of fatty acids palmitic, stearic, oleic and linoleic were quantified following the procedure described by Delgado-Chavero *et al.* (2013). To separate and transfer FAMEs to the spectrometer, a gas chromatograph Agilent 7890A GC System provided with a capillary column (30 m × 0.25 mm ID and 0.25 µm thickness) was used (Agilent Technologies, Waldbronn, Germany). Helium was the carrier gas. The injector temperature was 280 °C and the detector was 300 °C. The isotope ratio mass spectrometry analyses were done on a Hydra 20-20® model (SerCon Ltd, Crewe, UK) with continuous flow gas source, equipped with an electromagnet, a combustion interface and a Nafion membrane to retain water from the combustion product. The combustion tube temperature was 860ºC. As standards three commercial FAMEs (methyl-hexadecanoate, methyl-heptadecanoate and methyl-heneicosanoate (Sigma-Aldrich Co., St. Louis, MO, USA) were used. As reference material Iberian pig

subcutaneous fat, characterised by the stable isotope laboratory of the University of Salamanca and by our own laboratory, was used as control. The isotopic value obtained is expressed in terms of "δ", which represents the excess, typically heavy isotope, in a sample relative to a gas reference, ‰ units, referred to PDB (Pee Dee Belemnite; international reference data δ13C). A regression line of the three internal standards analysed along with the unknown samples was used to normalise the measured values. The Goodman and Brenna formula (Goodman & Brenna, 1992) has been applied to obtain the FAMEs isotopic value discounting the contribution of methylating agent.

Statistical analysis

The data were analysed as a randomized design using the general linear model (GLM) procedure contained in SAS v. 9.3 (SAS Inst. Inc., Cary, NC, USA). A Duncan's multiple range test was performed to test differences on the rearing regime effect. Differences between means were considered statistically significant when $p<0.05$. The linear discriminant analysis was performed using the DISCRIM procedure (SAS v. 9.3) on the samples collected over the first three seasons including the following additional variables: the sums of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and unsaturated (UFA) fatty acids, the ratio SFA:UFA, the ratio between the four δ-values of the fatty acids methyl esters (δC16:0/δC18:0, δC16:0/δC18:1, δC16:0/δC18:2, δC18:0/δC18:1, δC18:0/δC18:2, δC18:1/δC18:2) and the ratio gamma:alpha tocopherol (γ-toc/α-toc). The resulting linear discriminant functions obtained from data of S1-S3 were used to predict the feeding system in those samples collected over the fourth season. Several alternatives were considered with respect to the variables involved in the discriminant analysis and to the feeding categories. In the first case, three discriminant analyses with the variables of each method alone (FA, TOC and ISO) were performed, secondly an analysis of the methods two by two (FA+TOC, FA+ISO and TOC+ISO) and, finally, all the variables were included (FA+TOC+ISO). Regarding the feeding categories, the samples were classified into one of the four traditional systems (BE, RE, CA and CE), but prediction results were also obtained for joined samples from feeding systems based on acorn (BE plus RE) *vs* joined samples from systems non-based on acorn feeding (CA plus CE).

Results and discussion

Traceability is one of the most important topics nowadays in the food policy and strategy in most of the countries. It is needed not only to ensure that all food products are safe for citizens but also allows providing accurate information to the public, minimizing the disruption of trade (EC, 2007). In this context, an example of strategy for food authentication should be the one based in the differentiation of free-range animals from those fed in intensive conditions such as happen in the Iberian pig production. Taking into account the fact that some analytical methods have shown higher rate of feasibility for distinguishing feeding background of Iberian pigs than others, their classification ability (alone or in combination) in practical conditions on unknown samples (S4) was checked in the present study.

Table 2 presents the mean values of the main fatty acids percentage, FAMEs isotopes and concentrations of alpha and gamma tocopherols (μ/g) over seasons 1 to 3 (reliable database) and season 4 (data for classification) according to the rearing regime. Differences between variables according to the feeding background were considered statistically significant with a $p<0.0001$. Regarding the fatty acid profile, there was a clear gradation of the two main saturated FA (C16:0, C18:0) from CE to BE samples in S1-S3 seasons, with intermediate values for CA and RE groups, as expected (Sanabria *et al.*, 2013). The gradation was opposite in oleic acid for S1-S3 being more abundant in BE following by RE, CA and CE in line with similar results observed previously in pigs receiving a reliable feeding (Sanabria *et al.*, 2013). However, oleic acid and stearic proportions were similar in S4 for BE and RE groups. These clear trends from the most extensive system to the more intensive one was not observed in linoleic acid because the RE group had greater proportions than the other groups in S1-S3, whereas in S4 the greatest proportions were found in BE and CA groups. In both cases, CE presented lesser C18:2 values than the other groups. C18:2 is an essential fatty acid that cannot be synthetized in the pig organism, so its tissue proportion depends on the feeding system, its metabolic utilization, or the proportion of other fatty acids (López-Bote *et al.*, 1999). Concerning the second selected analytical method, there was not an evident trend for the FAMEs δ^{13} mean values, neither in S1-S3 nor in S4 as observed before (Delgado-Chavero *et al.*, 2013). Only CA system presented the greatest values for the four variables and RE system tended to have lesser than the others. Tocopherol composition (the last selected analytical method) again showed a graduation in mean values according to the duration of the extensive system, especially in γ-toc with very clear greater values in BE followed by RE, CA and CE groups. The differences were not so obvious in α-toc content; however CE group had lesser values than the others in S1-S3 and S4 seasons. Similar mean values have been reported in previous studies using tocopherols as indicators of the feeding background (Rey *et al.*, 2006, 2013).

Table 3 shows the classification of fat samples belonging to the fourth season by feeding system, according

Table 2. Rearing regime average values of main fatty acid percentages, δ-values of the fatty acids methyl esters and concentration of alpha and gamma tocopherol (μ/g) over seasons 1 to 3 and in season 4

Variable	Rearing regime means[1]							
	S1–S3 training set				S4 validation set			
	BE	RE	CA	CE	BE	RE	CA	CE
Fatty acids								
C16:0	19.92^{d}	20.48^{c}	21.62^{b}	22.75^{a}	19.36^{d}	19.88^{c}	22.06^{b}	23.17^{a}
C18:0	9.03^{d}	9.67^{c}	11.24^{b}	12.06^{a}	8.58^{c}	8.90^{c}	11.04^{b}	12.19^{a}
C18:1	55.97^{a}	53.71^{b}	51.60^{c}	50.65^{d}	56.97^{a}	56.41^{a}	51.32^{b}	50.40^{c}
C18:2	8.97^{b}	9.67^{a}	8.96^{b}	7.86^{c}	8.82^{a}	8.41^{b}	8.91^{a}	7.69^{c}
Isotopes								
δ^{13}C C16:0	-26.71^{c}	-24.92^{b}	-24.37^{a}	-24.79^{ab}	-26.75^{d}	-25.71^{b}	-24.95^{a}	-26.28^{c}
δ^{13}C C18:0	-24.08^{c}	-22.42^{b}	-21.89^{a}	-22.29^{ab}	-23.88^{d}	-22.71^{b}	-22.14^{a}	-23.48^{c}
δ^{13}C C18:1	-27.82^{c}	-25.95^{b}	-24.91^{a}	-25.18^{a}	-27.30^{d}	-26.22^{c}	-24.33^{a}	-25.63^{b}
δ^{13}C C18:2	-33.30^{d}	-31.67^{b}	-30.94^{a}	-32.38^{c}	-32.76^{c}	-32.34^{b}	-30.80^{a}	-33.52^{d}
Tocopherols								
α-toc	9.84^{ab}	10.34^{a}	9.22^{b}	5.82^{c}	14.30^{b}	15.77^{a}	10.29^{c}	6.78^{d}
γ-toc	1.69^{a}	0.97^{b}	0.43^{c}	0.40^{c}	1.68^{a}	1.11^{b}	0.59^{c}	0.29^{d}

BE: free-range with *ad libitum* intake of acorn and pastures. RE: free-range with ad libitum acorn and pastures plus commercial feed. CA: open-air system feeding with commercial feeds besides of the grazing of grass or stubble. CE: standard feeding with commercial diet. δ ‰ units, excess of heavy isotope in a sample referred to the international reference PDB. Within the same row different superscript letters indicate statistically significant differences (p<0.0001). [1] p-values of rearing regime effect were always significant (p<0.0001)

Table 3. Classification of the fourth season (S4) by production system (%) according to linear discriminant functions based on data from season 1 to 3 by each method alone.

Production system	BE	RE	CA	CE	Overall success
Fatty acids					60.1
BE	93	6	1	0	
RE	57	33	10	0	
CA	0	5	3	92	
CE	0	26	4	70	
Isotopes					49.7
BE	28	63	9	0	
RE	12	43	18	27	
CA	0	0	85	15	
CE	0	6	24	70	
Tocopherols					49.3
BE	46	54	0	0	
RE	27	57	16	0	
CA	0	21	39	40	
CE	0	2	34	64	

BE: free-range with ad libitum intake of acorn and pastures. RE: free-range with *ad libitum* acorn and pastures plus commercial feed. CA: open-air system feeding with commercial feeds besides of the grazing of grass or stubble. CE: standard feeding with commercial diet

to the linear discriminant functions obtained from data of each method (S1-S3) (FA, ISO and TOC). It was assumed that the classification of batches certified by the control organisations for the samples of the fourth season was true (Table 1). The percentages of correct assignation of the FA method for BE and CE samples were very high but the results were not satisfactory for RE (57% were classified as BE) and for CA (the method was unable to distinguish this category from CE). ISO method allowed differentiating CA as well as CE feeding systems, with not adequate results for BE and RE samples. Finally, TOC method showed intermediate values (from 39% to 64%) of correct predictions into the four feeding categories. The overall success rate was 60.1% for FA, 49.7% for ISO and 49.3% for TOC. These results were lesser than those reported before in pigs of known rearing regimes (García-Casco *et al.*, 2013). These authors reported averaged success rates into four categories of feeding of 74% and 69% for TOC and ISO methods, respectively, and FA had lesser percentage of correct classification than ISO or TOC determination. The results of the present study could be explained because some batches were not correctly classified by the control organisations. Table S1 [suppl.] shows the classification of each individual batch in the S4. This analysis points out as some of the batches included in this study, approximately a 25% of the samples certified officially as BE, did not meet the minimum requirements for the content of tocopherols or stable isotopes to be classified in this category.

All the 34 samples of the batches BE9, BE10 and BE11, with available information about supplementation with commercial feeds during the free-range period, were classified as BE by FA method, while ISO and TOC allocated them correctly in the RE category. BE4 batch was mostly predicted as RE by the three methods, therefore this consensus advised the reclassification as RE. In other four batches (BE3, BE5, BE7 and BE8) doubts may arise on the official classification in spite of the categorical prediction as BE by FA. Again, additional and feasible information provided by the farmer to the authors about free-range period without commercial feed supplementation in batch BE7 and the same pattern of results observed in BE3 as in BE7, support the permanence of both in the BE category. However, BE5 and BE8 batches, which had opposite classification results with ISO and TOC methods, lead to consider the possibility that these batches belong to RE category. There is not additional information in the field allowing make assumptions about management and feeding in BE5 and BE8. But the scarce availability of acorn in that geographical area at that time, makes it feasible to suppose an insufficient weight gain based exclusively in acorn and pastures and, as a consequence, the supplementation with commercial feeds during the last stage of the free-range period.. It is common to use commercial feeds containing high-oleic raw materials in the final stages prior to slaughter, even in immediately periods prior to the *Montanera*. As a result of this feeding, the final fat samples usually have high oleic acid proportions that allow the samples to be categorized as BE in an analysis with the FA method. This effect on oleic acid proportion of RE was observed in the mean values of samples from S4, as was commented before (Table 2). This is one of the most important reasons that explain the difficulty for a feasible distinguishing of BE and RE categories by some analytical methods. This analytical limitation is also preceded by the complexity and high cost to ensure, through rigorous field controls in extensive wooded areas, the absence of commercial feeds supplementation. Additionally, it is also of interest to point out that the three methods considered CE1 samples in a wrong category, as CA (ISO and TOC) and even as RE (FA). Therefore, the CE1 was reclassified as CA because pigs belonged to an industry interested only in labeling the products as BE or CE, even if the production system were close to the characteristic of CA feeding.

Table 4 contains the prediction ability in fat samples from the fourth season according to linear discriminant functions of each method after the modifications of category assigned by control organisations (BE4, BE5, BE8, BE9, BE10 and BE11 as RE; and CE1 as CA). The ISO and TOC analysis had a 70% of success rate into the four categories of pigs, in turn with previous prediction results of these methods (García-Casco *et al.*, 2013), while fatty acid analysis showed 40.5%, attributable to the use of high-oleic commercial diets as mentioned above.

The effect of two methods combination on the prediction ability is also shown in Table 4. The combination of ISO variables with FA increased substantially the deficient results obtained with FA alone for the RE and CA categories but, on the other hand, there was an important decrease of correct predictions for the BE system, with 36% classified as RE. The combined analysis including FA and TOC variables improved slightly the classification of CA samples (only 13% of correct predictions, with 77% placed in CE). ISO+TOC was the most successful combination, showing the highest rate of mistakes in the BE samples with 25% of them classified as RE. The overall success was 67.2% for FA+ISO, 61.8% for FA+TOC and 84.1% for ISO+TOC. The overall success with the discriminant analysis including all the variables of the three methods was 80.4%. There is not previous information on the combination of these analytical methods for distinguishing the feeding background of Iberian pigs, so these results show the importance of using different methods to improve the success rate of classification. Figure 1 represents the distribution (with two canonical discriminant functions) for the joint analysis with the best overall prediction (ISO+TOC), after the reclassification. There is not a clear spatial separation between BE and RE areas, and 15 samples of each system are embedded

Table 4. Classification of samples (% assigned correctly) from fourth season into four feeding categories according to linear discriminant functions using each method alone or two-by-two method combinations, after re-classification of batches BE4, BE5, BE8, BE9, BE10, BE11 and CE1.

	BE	RE	CA	CE	Overall success
Fatty acids	98	18	5	100	40.5
Isotopes	44	64	84	100	69.6
Tocopherols	83	73	45	83	68.9
Fatty acids + Isotopes					67.2
BE	63	36	2	0	
RE	29	61	10	0	
CA	0	13	66	21	
CE	0	0	0	100	
Fatty acids + Tocopherols					61.8
BE	98	2	0	0	
RE	30	64	6	0	
CA	0	10	13	77	
CE	0	0	0	100	
Isotopes + Tocopherols					84.1
BE	73	25	2	0	
RE	12	81	2	5	
CA	0	0	91	9	
CE	0	0	0	100	

BE: free-range with *ad libitum* intake of acorn and pastures. RE: free-range with *ad libitum* acorn and pastures plus commercial feed. CA: open-air system feeding with commercial feeds besides of the grazing of grass or stubble. CE: standard feeding with commercial diet

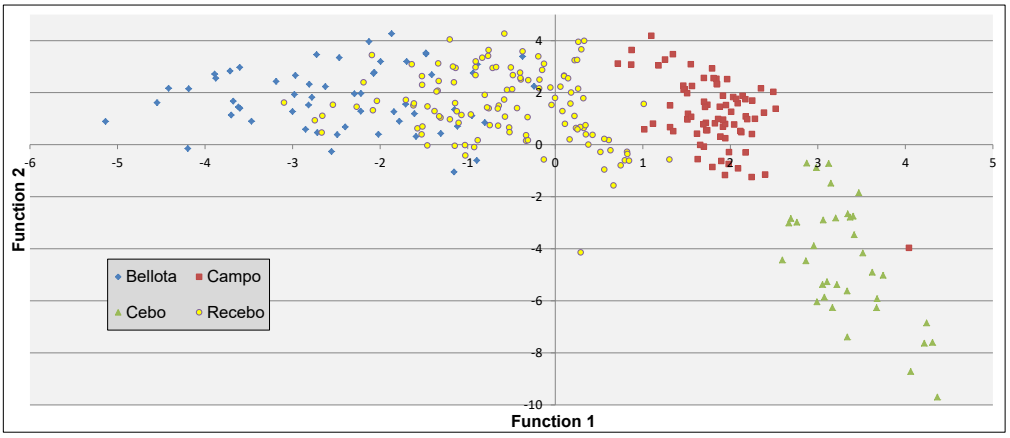

Figure 1. Distribution of the samples according to rearing regime, represented with the canonical discriminant functions performed by Isotopes+Tocopherols variables, after reclassification of batches BE4, BE5, BE8, BE9, BE10, BE11 and CE1

in the opposite area, but CA and CE groups are well-defined. As it has been shown with some of the batches included in this study, approximately a 25% of the samples certified officially as BE did not meet the minimum requirements for the content of tocopherols or stable isotopes to be classified in that category. The difficulty of separating clearly BE and RE was officially resolved with a new legislation that eliminated the RE category (BOE, 2014). However, the difficulty does not imply that in many other cases this distinction is not feasible. Only in a few batches there could be uncertainty about the type of feeding. The quantification of tocopherols, especially gamma-tocopherol, constitutes a strong differentiating factor for the distinction between these two categories. Moreover, the correct classifications for pigs raised on different feeding systems can be extended to dry-cured products by applying alpha and gamma-tocopherol content (Rey *et al.*, 2014). The addition of stable isotopes to the quantification of tocopherol would further increase the detection of many RE pigs incorrectly certified as BE.

The distinction between CA in autumn-winter, with pasture available, and CE seems possible with a high success rate with ISO technique. The $^{13}C/^{12}C$ ratio in plants depends on the photosynthetic mechanism they used. The C3 plants, which use only the Calvin cycle for fixing atmospheric carbon dioxide, have $\delta^{13}C$ values in the range of -20/-37 ‰, whereas C4 plants, such as corn, typical of tropical areas, which use a process in two stages to fix CO2, have $\delta^{13}C$ values in the range of -12/-16 ‰ (O'Leary, 1988). Then, the values of the $^{13}C/^{12}C$ ratio, and hence δ13C, in the pastures of temperate and cold zones (with abundance of C3 plants) are lower than those of commercial feeds with C4 plants added, such as corn, and these values are reflected in the tissues of animals fed with one or other type of plants (Minson *et al.*, 1975; Tieszen *et al.*, 1979).

The differentiation is even better if TOC is also considered, although the presence of alpha-tocopherol in the fat may be caused by the addition of this element in the commercial feeds, which has very likely occurred in the CE1 batch (mean α-toc = 9.94 μ/g). The consumption of small amounts of acorn in CA conditions is suggested by the high value of gamma-tocopherol (γ-toc = 0.84) found in CA5 batch. The normative requirements for CA are set out only relating to the availability of space and the slaughter age, therefore this category does not imply consumption of pastures (BOE, 2014). The feeding in large fenced areas during the long summer (May to September) in the southwest of the Iberian Peninsula, exclusively with commercial feed consumption (without availability of pasture), will probably cause identical analytical results for CA and CE categories, making its differentiation very questionable.

The prediction results for joined feeding categories based on acorn (BE plus RE) *vs* non-acorn categories (CA plus CE) were much more accurate, as it is summarized in Table 5 for the seven discriminant analysis. With the exception of ISO method, the overall percentage of correct predictions was over 90%, almost 95% for FA+TOC and ISO+TOC. This rate of success with methods combination was similar to that reported for the individual use of TOC (98%) in pigs with reliable feeding (García-Casco

Table 5. Classification of samples (% assigned correctly) from fourth season into two (acorn *vs* non-acorn) or three feeding categories (acorn (BE+RE), CA and CE)[1] according to the linear discriminant function using each independent method or two by two, or three method combinations.

	FA	ISO	TOC	FA+ISO	FA+TOC	ISO+TOC	FA+ISO +TOC
Two categories[2]							
Acorn	97.0	81.4	93.0	94.5	94.0	92.0	91.0
No-Acorn	87.6	95.9	91.7	90.9	95.9	99.2	98.3
Overall success	93.4	86.9	92.5	93.1	94.7	94.7	93.8
Three categories							
Acorn	95	64	89	89	89	84	84
CA	9	87	55	70	21	91	81
CE	100	100	83	100	100	100	100
Overall success	73	74	79	85	73	88	85

[1] BE: free-range with *ad libitum* intake of acorn and pastures. RE: free-range with ad libitum acorn and pastures plus commercial feed. CA: open-air system feeding with commercial feeds besides of the grazing of grass or stubble. CE: standard feeding with commercial diet. [2] Acorn: BE plus RE. No-Acorn: CA plus CE. FA, ISO, TOC: Fatty acids, isotopes and tocopherols analyses, respectively

et al., 2013). In the present study, ISO and FA improved their classification ability into two categories when they were combined with TOC. When three feeding categories were considered grouping BE and RE, the success rate reached an 88% with the combination of ISO and TOC analyses together, although it should be mentioned again the necessary presence of grass in the diet of the pigs to maintain this level of success in the prediction. Figure 2 presents the distribution of samples with two canonical functions with the ISO+TOC analysis for both cases. There is not previous information of the classification ability into two feeding categories by the combination of different methods, and according to the results of the present study, the distinction between the extensive feeding based on acorn (indistinctly BE or RE) and the feeding based on commercial feed (CE or CA) is entirely possible. The incorporation of the sums and ratio of FA and ratios between δ-values and tocopherols to the set of simple variables to performance the discriminant analyses, improved slightly the predictions, without adding undesirable extra effort for a routine procedure.

The analyses and results of this paper always refer to a single prediction, sample by sample, from each animal. However, the implementation by industry of analytical methods is always performed on a pool of samples, *i.e.* through a unique analysis of the samples obtained in a proportion of animals of the batches. As it has been already noted in García-Casco *et al.* (2013) the consideration of the batch as an experimental unit would increase the success rate very significantly in all cases and the usefulness of this type of analysis. The combination of two methods, preferably TOC plus ISO, imposes an extra cost hardly acceptable if all the production batches are contrasted, but its application could be very interesting for verification in case of disputes in buy and sell agreements of Iberian pigs and should be considered by authorities.

In summary, the present study raises the suitability of the analytical methods (fatty acid profile, tocopherols content and stable isotope ratios) to predict the feeding system in Iberian pigs, through the classification of commercial batches by official control bodies. Moreover, the combination of two techniques in a discriminant analysis can improve the predictions achieved by each one individually. In that sense, the combination TOC + ISO obtained the most accurate results. This combination allows distinguishing free-range pigs from those fed free-range and given supplemental feeds, as well as those fed with feed in extensive conditions from those fed exclusively with mixed diets. The application of these methods avoids undesirable misclassifications. The distinction between

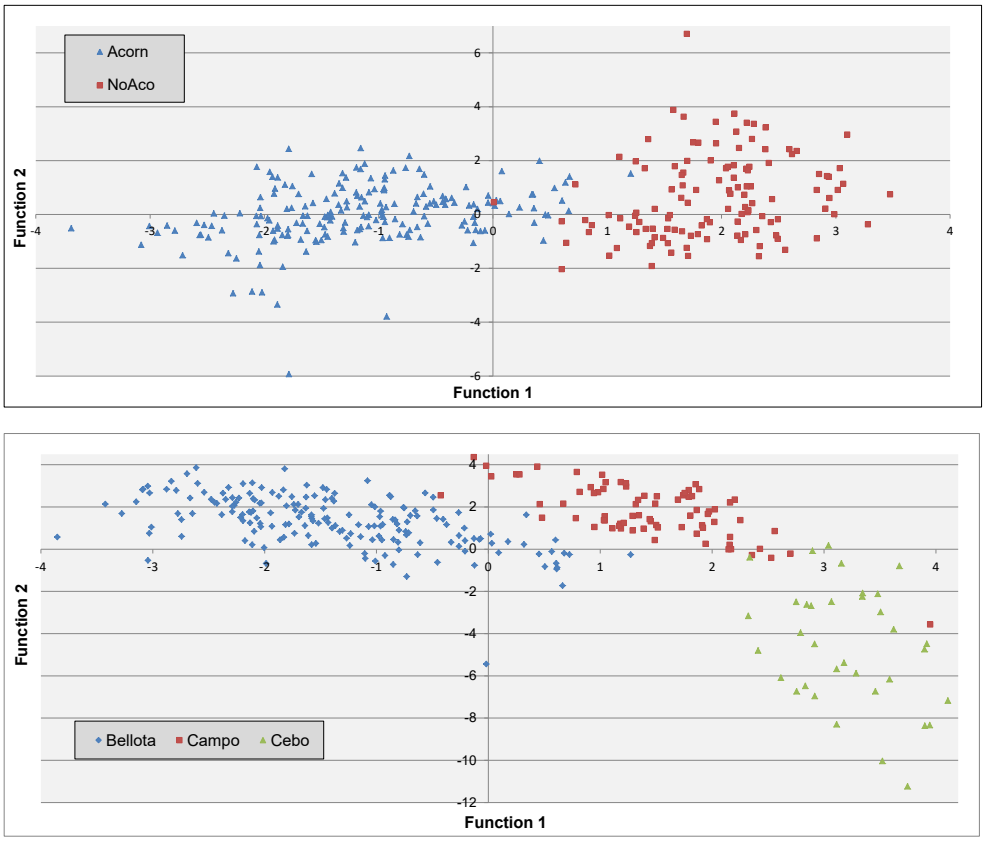

Figure 2. Distribution of the samples according to two (acorn *vs* no-acorn) (a) or three (*bellota, campo* and *cebo*) (b) rearing regimes represented with the canonical functions of the discriminant analysis performed with Isotopes+Tocopherols variables

two categories (acorn *vs* non-acorn) is extremely effective with all three methods and their combinations. Therefore, the use of the analytical methods proposed in this study as a complementary tool to the required quality controls is highly recommended.

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