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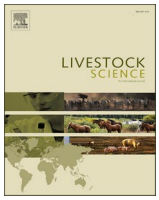

In vivo authentication of Iberian pig feeding regime using faecal volatilome information

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HIGHLIGHTS

- Faecal volatilome information provides useful information for an *in vivo* authentication of Iberian pig feeding regime.
- The current commercial categories of Iberian products in terms of animal feeding regime were correctly predicted, obtaining good classification results.
- Gas chromatography coupled to ion mobility spectrometry could become an effective tool as an *in vivo* control for the improvement of Iberian product certification.

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ABSTRACT

Nowadays, people are increasingly interested in the food they consume. Authenticity and natural origin are amongst the most valued issues of food products by society. Although various national and international laws have been created for the regulation of labelling and trade of food, unfortunately, they are often not effective in avoiding food product fraud. The Iberian pig and the cured products obtained with this breed have a great international reputation due to their high quality and added value. However, the authentication of these pigs feeding regime is sometimes difficult. Therefore, the objective of this study was to use faecal volatilome information to differentiate the different feeding regimes which determine the final commercial category of Iberian products. Individual faeces samples were sampled on 10 farms from 133 Iberian pigs to evaluate their volatilome through gas chromatography (GC) coupled to ion mobility spectrometry (IMS). The intensity of GC-IMS plot features were extracted and chemometric tools were employed to develop two different models: one, focused on the discrimination between acorn-fed (completely natural diet grazed) and feed-fed samples, and another one for commercial category classification. Both models were carried out in duplicate, using spectral fingerprint information and a different approach studying specific markers. Good classification rates were obtained in both models: 92,3% and 96,3% were the rates obtained in acorn-fed vs feed-fed model with fingerprint and specific markers information, respectively; and the same classification success was also achieved with both approaches in the second model, focused on commercial category classification. The misclassified samples in both models, which belonged to acorn-fed pigs, may be related to the diet heterogeneity of these animals and the differences in natural resources foraged. The results of the present study highlight GC-IMS as an useful tool to carry out an *in vivo* authentication of Iberian pig feeding regime and the subsequent commercial category, as well as to avoid labelling fraud. Further studies including larger number of samples are needed in order to obtain more complex models to classify very different samples.

1. Introduction

Nowadays, interest in food authenticity in society has increased and become a priority for the whole food industry, implying all levels of the production and distribution process (Hong et al., 2017). Fraud constitutes a problem in the food sector which leads to negative economic

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impacts and potential risks in food security (Hassoun et al., 2020).

Recently, a new habit has emerged between consumers based on the consideration of healthier, safer, more natural, environmentally friendlier and higher quality products coming from differentiated manufacturers in comparison to conventional foods (Hong et al., 2017; Rey and López-Bote, 2014). This is the case of organic, extensive or grass-fed labelling, and certification of food products from livestock farming. As a consequence of this added value, consumers agree to pay more for such products (Cossignani et al., 2019; Rey and López-Bote, 2014) and this extra price over conventional products explains the frequent appearance of fraud cases (Capuano et al., 2013).

Representative cases of these high added-value products are those obtained from the Iberian pig, which has been traditionally reared in the southwestern Iberian Peninsula (Spain and Portugal), exploiting its adaptation capacity to this environment and to the feeding resources from the *dehesa* agroecosystem (Lopez-Bote, 1998; Rodríguez-Estévez et al., 2009). Iberian products have a considerable international reputation due to their high quality (organoleptic and nutritional) and added value (Fernández-Cabanás et al., 2007; Ventanas et al., 2005). However, although Iberian pig breeding has always been related to the *dehesa* (Rodríguez-Estévez et al., 2009), current productive systems are deeply different in terms of animal feeding and freedom of movement. Thus, most Iberian pigs are reared under totally intensive systems where compound feed constitutes their only feeding source; but there are also extensive (outdoor) systems where pigs have a free natural diet based on the foraging of acorns, pastures and other natural resources throughout the final stage of the productive cycle (Rodríguez-Estévez et al., 2012), without allowing any supplementation for at least the last two months of fattening (Ministerio de Agricultura Pesca y Alimentación, 2014; Rodríguez-Estévez et al., 2009). This finishing system is called "montanera" in Spanish (meaning pannage). These two models produce two types of cured hams from Iberian pigs called "feed ham" and "acorn ham" ("de cebo" and "de bellota" in Spanish), respectively. A Spanish regulation summarises the quality characteristics that Iberian pig production must ensure, both at field and on finished products; and also regulates labelling, commercialisation, traceability and certification (Ministerio de Agricultura, Pesca y Alimentación, 2014). The field inspections of Iberian pig production are mainly based on visual observations and traceability documents. In this regard, there is no standardised procedure to carry out *in vivo* authentication of Iberian pig production regarding their feeding regime. The latter aspect mainly defines quality differences between products (Carrapiso et al., 2003) and, hence, is susceptible to fraud. Feeding regimes, together with breed purity, determine the denomination and price of commercialised Iberian products. Therefore, an inadequate certification has a great impact on consumers and producers, implying important economic repercussions.

Faced with the need to protect consumers and producers, the development and research of accurate analytical techniques which contribute to the authentication of food products is crucial (Alonso et al., 2008; Ballin, 2010; Hassoun et al., 2020; Hong et al., 2017).

Although significant efforts have been made in recent years to develop methodologies focused on the differentiation of Iberian pig feeding (Martín-Gómez et al., 2019), most of these were focused on the evaluation of final products, mainly dry cured ham (Arroyo-Manzanares et al., 2018; Bayés-García et al., 2016; Carrapiso et al., 2015; Del Pulgar et al., 2011; Santos et al., 2004); or samples collected at the slaughterhouse, such as subcutaneous fat, muscle or liver tissue (Alonso et al., 2008; Arce et al., 2009; Gamero-Pasadas et al., 2006; García-Olmo et al., 2009; González-Martín et al., 2001; Hernández-Matamoros et al., 2013; López-Vidal et al., 2008; Rey et al., 2006; Rey and López-Bote, 2014; Viera-Alcaide et al., 2008). However, there is a lack of consideration towards *in vivo* collected samples, which would allow a complementary control of pigs feeding at field.

The volatilome constitutes the set of volatile organic compounds (VOCs) produced by an organism (Amann et al., 2014; Filipiak et al., 2016). These are low-mass molecular substances characterised by low boiling points and high vapour pressures (Ebert et al., 2016; Tejero Rioseras et al., 2017), which can therefore be monitored by non-invasive methods and easier samplings compared to other metabolites (Sinha et al., 2017; Singh et al., 2018). On the other hand, faeces are a complex biological material, interesting for their easy and accessible collection as well as the information they provide about physiology (Rodríguez-Hernández et al., 2020). In this regard, faecal volatilome has been widely employed to study different fields of animal science: from reproduction (Karthikeyan et al., 2013) and animal health (Rodríguez-Hernández et al., 2021), to evaluation of diet composition (Pérez-Calvo et al., 2019; Recharla et al., 2017).

The objective of this study is to evaluate faecal volatilome analysis as a possible tool to carry out an *in vivo* authentication of the Iberian pig feeding regime.

2. Materials and method

2.1. Animals and farms

10 Iberian pig farms and 133 animals were included in the present study. Pig farms were classified according to the Spanish regulation for the Iberian pig feeding regime (Ministerio de Agricultura Pesca y Alimentación, 2014). Three different types of farms were differentiated: (i) acorn or "Bellota" (A) farms, where pigs were raised on *dehesa* farms during the last part of the productive cycle with a feeding regime based on acorns of evergreen oaks (*Quercus rotundifolia*) and grass, without allowing any supplementary feed; (ii) feed or "Cebo" (F) farms, where pigs were stabled and fed with compound feed; and lastly, (iii) outdoor feed or "Cebo de Campo" (OF) farms, mixed productive systems of pigs raised outdoors, with a feeding regime depending on feed although animals could slightly forage natural resources. The animals included in the present study were fattening Iberian pigs in the last stage of the productive cycle (approximately from 100 to 160 kg live weight). Regarding the breed purity of the pigs, it varied between 100% Iberian and 50% (Duroc crossed), depending on the farm. Most of the acorn-fed pigs were controlled by inspectors of the Protected Designation of Origin Los Pedroches.

2.2. Sample collection and processing

A total of 133 fresh faeces samples were collected from Iberian pigs reared on farms with different feeding regimes (Table 1). Sample collection was carried out monitoring animals until spontaneous defecation occurred; thus, each sample obtained was assured to have come from individual animals. Faeces samples were collected directly from the ground just after defecation, discarding the part of the sample in contact with soil material to avoid any possible contamination. Samples were transported to the laboratory in refrigeration boxes and stored at -18 °C until processing and analysis.

Faeces samples were thawed overnight the day before analysis and processed early in the morning. A precision balance was employed to weigh 1.5 g of fresh faeces in a 20 mL glass vial, which was subsequently closed with a metallic cap and a silicone septum. Analysis of faecal volatilome was always carried out during the same day of sample processing, to prevent possible sample degradation.

Table 1
Distribution of samples according to farm classification and models.

Farm classification	Number of sampled animals	Number of sampled farms	Model 1	Model 2
Acorn (A)	74	6	74	74
Outdoor feed (OF)	26	2	59*	26
Feed (F)	33	2		33

*In model 1, OF and F samples were considered in the same group (Feed-fed).

2.3. Instrumentation and software analysis

Faecal volatilome analysis was performed using the commercial ion mobility spectrometer (IMS) FlavourSpec® (Gesellschaft für Analytische Sensorsysteme mbH (G.A.S.), Dortmund, Germany). A heated splitless injector enabled direct sampling of the headspace using a 2.5 mL Hamilton syringe containing a 51 mm needle. The device was equipped with an autosampler (Combi Pal, CTC Analytics AG, Zwingen, Switzerland) and fitted with a fused silica 30 m × 0.32 mm ID capillary column with a non-polar 0.5 μm HP-5 stationary phase (5%-Phenyl)-methylpolysiloxane (Agilent, Santa Clara, California, United States of America). The ionisation source of the IMS was tritium, which provided a radiation energy of 6.5 KeV. VOCs travel through the 10 cm drift tube and generate an electrical signal by colliding with a Faraday plate with an intensity proportional to the concentration in the sample. IMS instrument data were acquired in positive mode and evaluated using LAV® 2.1.1 software supplied by G.A.S.

The GC-IMS method employed during faeces analysis was optimised according to the following parameters: amount of sample, incubation temperature, sample heating time, Gas Chromatography (GC) column temperature, drift tube temperature, carrier gas flow rate and drift gas flow rate. Optimisation was based on the evaluation of features found in GC-IMS maps after parameter modifications to achieve optimal resolution and sensitivity. Optimisation information is shown in Table 2. The values of intensity, retention time and drift time of the proton-bound dimer of 6 ketones were evaluated daily during the period of study to monitor the condition of the instrument and ensure the reliability of the results (Jurado-Campos et al., 2021).

MATLAB® software (The Mathworks Inc., Natick, MA, USA, 2007), its plugin PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA), and SIMCA® 14.1 software (Umetrics) were employed as statistical tools for data treatment and chemometrics. Moreover, VOCal software (G.A.S.) was employed to tentatively identify unknown features as potential markers.

2.4. Chemometrics and statistical analysis

Due to the three-dimensional nature of GC–IMS measurements, large quantities of complex data were obtained. In GC-IMS plots, the Y axis represents the retention time in the chromatographic column (in s), the X axis represents the drift time in the drift tube (in ms), and the Z axis the intensity value (in V) of each ion. Chemometric techniques were employed to reduce variable dimensions and find combinations that describe main trends in the data obtained after analysis. Two different approaches were considered during data treatment: one, based on the study of the complete spectral fingerprint, and a second consisting of the processing of individual markers that appeared in the GC-IMS plot. Before data treatment, all spectra obtained were aligned employing as reference an individual feature present in every sample.

For the first strategy, LAV® software was used to convert raw IMS data to .csv format. Only the map part with most features was selected due to the large amount of generated data: from 1150 to 2150 ms of drift time and from 196 to 1690 s of retention time. Afterwards, MATLAB® PLS Toolbox was employed to carry out Partial Least Squares - Discriminant Analysis (PLS-DA).

For the second approach, a matrix containing extracted intensities of 409 features in 133 samples was employed for the construction and validation of the final classification models. The intensity of these markers was obtained using LAV software and a matrix was built with all samples and all selected markers. SIMCA® software was used for data processing and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). In addition, the influence of markers used on the classification of samples was measured by their Variable Importance for the Projection (VIP). VIP constitutes a valuable component in the analysis of complex data such as metabolome or volatilome that allows the selection of unknown markers from multiple potential analytes which could be measured as biomarkers (Trivedi, 2012). This allowed the evaluation and selection of those features with a significant effect on sample classification. The different chemometric models in both strategies were built using 80% of the analysed samples (training set) and the remaining 20% was used for their validation (validation set). In both cases, a Principal Component Analysis (PCA) was carried out before discriminant analysis to explore data and detect possible outliers.

Subsequently, two different models were performed by duplicate (for both approaches: using fingerprint or individual markers). Firstly, a preliminary model was built to find differences between animals mainly fed with acorns and natural resources (acorn-fed), or with feed (feed-fed); while in the first group only samples from A farms were included, the second group was composed with samples from OF and F farms. Secondly, another model was built to study Iberian pig commercial categories in terms of animal feeding, defined in Section 2.1: A, OF and F. The distribution of samples for each chemometric approach is shown in Table 1. A slightly higher number of A samples was collected in comparison with the rest of the groups to cover the broad variability which can be found in the natural systems where these samples came from.

Table 2
Optimised method parameters and final selected values.

Parameter	Evaluated interval	Selected value
Sample weight	0,5, 1 and 1,5 g.	1,5 g.
Sample incubation temperature	40, 60 and 80 °C	60 °C
Sample incubation time	5, 10 and 15 min.	15 min.
GC column temperature	40, 45 and 60 °C	45 °C
Drift tube temperature	45, 50, 55, and 75 °C	75 °C
Carrier gas flow rate	1, 10, 15, 20 and 25 mL min^{-1}	Flow ramp: 1-20 mL $min.^{-1}$
Drift gas flow rate	150, 200 and 250 mL min^{-1}	150 mL $min.^{-1}$

3. Results and discussion

The chemometric models were built using features present in tridimensional GC-IMS plots: using the whole spectral fingerprint or individual markers visually selected (including tentatively identified compounds and other non-identified signals). The PCA did not find any outlier, thus all samples analysed were employed to build discriminant models. Fig. 1 shows a visual comparison between GC-IMS plots of the three groups of samples evaluated in the present study. A tentative differentiation might be suggested based on the intensity and quantity of signals, which seems to be higher in samples from group A and slightly different between OF and F samples. This differentiation based on visual aspect was subsequently evaluated via chemometrics.

3.1. Model 1: acorn-fed vs feed-fed

The objective of model 1 was to differentiate between a natural diet based on acorns and natural resources grazed (acorn-fed), and a feeding regime with feed as the main and basic diet component (feed-fed). Table 3 shows the results after applying acorn vs feed feeding model with the fingerprint approach to the 26 samples used for validation (20% of the total). Only two acorn-fed samples were incorrectly classified in the model, obtaining an overall classification success of 92.3% depending on the pigs' feeding regime. These two samples were classified as feed-fed samples instead of acorn-fed. The representation of this approach is shown in the score plot in Fig. 2.

On the other hand, Table 4 contains information about the second strategy to carry out Model 1, where individual marker information was employed to build the model. A classification success of 96.2% was obtained. The results were very similar to the fingerprint approach: while all feed-fed samples were correctly predicted by the model; only

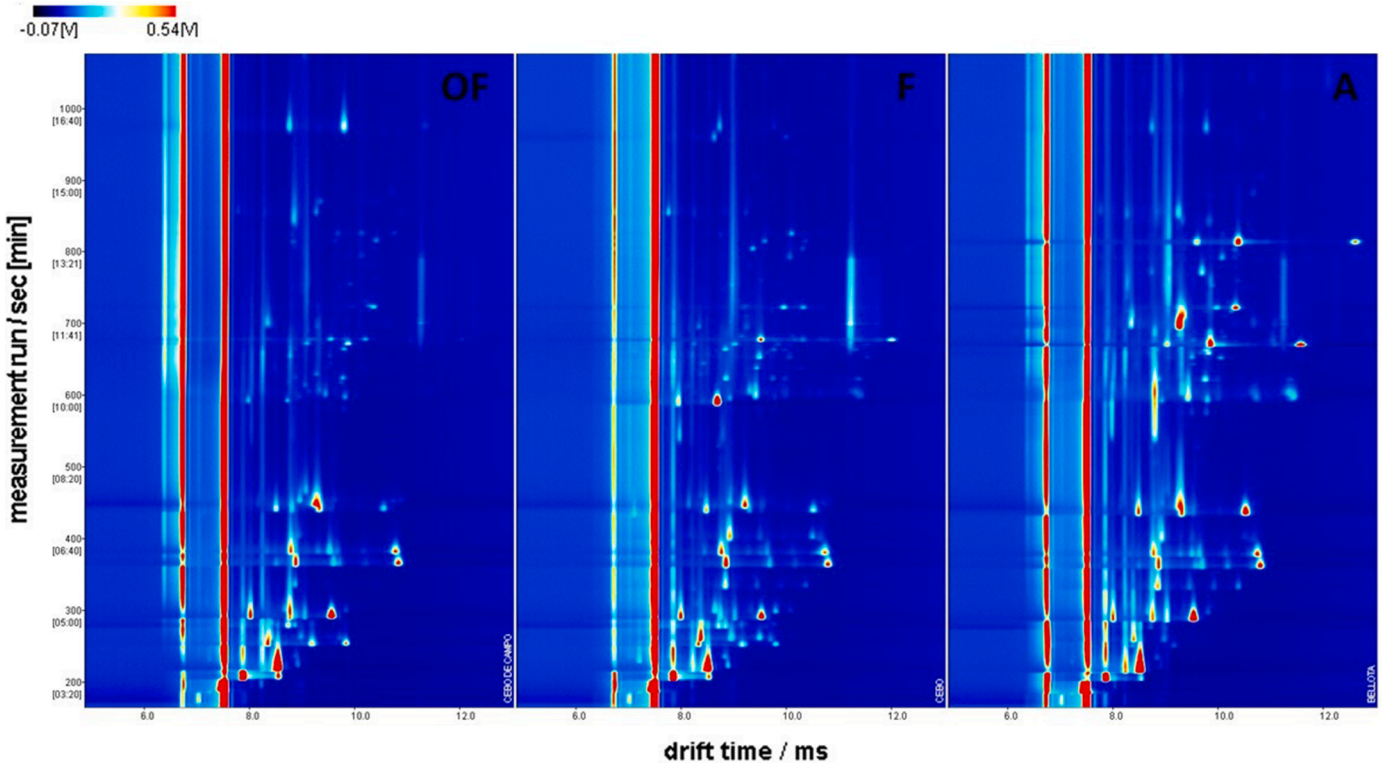

Fig. 1. Visual comparative between samples from "Cebo de campo" or "Outdoor feed" (OF), "Cebo" or "Feed" (F) and "Bellota" or "Acorn" (A) groups.

Table 3
Validation matrix for acorn-fed vs feed-fed classification (Model 1) using PLS-DA model with fingerprint information.

		Real classification	
		Acorn-fed	Feed-fed
Model prediction	Acorn-fed	13	0
	Feed-fed	2	11
	Total	15	11
	% category	86.70%	100%
	% total	92.30%	

one acorn-fed sample was incorrectly classified. This OPLS-DA model is graphically represented in Fig. 3. Some markers were suggested as significant for the classification of samples with VIP analysis: 2-pentanone, 2-methylbutan-1-ol, 2-hexanone, 2,3-butanediol and 2-heptanone.

Although not covered by the current Spanish regulation (Ministerio de Agricultura Pesca y Alimentación, 2014), this classification of samples distinguishing only between acorn and feed-fed animals has been widely used before to study the feeding regime of Iberian pigs (Alonso et al., 2008; Arroyo-Manzanares et al., 2018; Martín-Gómez et al., 2019). It is therefore important to consider that despite the efforts of the Spanish authorities, the labelling of Iberian products may be confusing for customers (Rodríguez-Estévez et al., 2012; Trienekens et al., 2009). In fact, some authors suggested a reduction in the number of product categories and its simplification in the two groups used in Model 1: "Acorn-fed" or "Bellota" and "Intensive-feeding (feed)" or "Cebo", in order to clarify the Iberian pig market (Carrasco and Duque, 2013). Taking into account that current consumers demand more complete information about the products they buy (Hassoun et al., 2020), this two-group classification would simplify the market of Iberian products, avoiding excessive information and categories which may be counterproductive.

In both spectral fingerprint and marker approaches all feed-fed samples were correctly classified and only a few acorn-fed samples were not. This finding could be very useful because there is no feed-fed animal classified as acorn-fed. The classification of a scarce number of acorn-fed samples as feed-fed could be explained by looking at a previous research (Rodríguez-Estévez et al., 2009) which studied the feeding behaviour of Iberian pigs on the *dehesa* through *in situ* observations: a considerable variation of the daily intake of acorns and grass (weight and proportion) was found, which must result in a large diet heterogeneity for acorn-fed samples due to differences in natural resources foraged. This factor may be associated with the error percentage of Model 1 and the incorrect classification of a few acorn-fed samples. In addition, the homogeneity obtained with a diet based on feed is not comparable with the heterogeneous diet resulting when foraging natural resources on the *dehesa*, where each animal can express their dietary preferences (Rodríguez-Estévez et al., 2012, 2009). This heterogeneity has also been found in volatilome analysis of cured Iberian hams from pigs fed with acorns (Martín-Gómez et al., 2019).

3.2. Model 2: classification by commercial category

Model 2 aimed to classify faeces samples following the current commercial categories for Iberian products according to Spanish regulation: groups A, OF and F. Sample classification obtained using fingerprint information is presented in Table 5. While all samples from groups OF and F were correctly classified, two samples from group A were incorrectly classified in group OF. The final classification rate obtained for the fingerprint model was 92.3%. The score plot of the fingerprint approach is shown in Fig. 4.

The use of specific marker information in Model 2 slightly increased classification success compared to fingerprint strategy. The results were very similar to the fingerprint approach; only one sample from group A was incorrectly classified in group OF, obtaining a 96.2% overall success (see Table 6). However, OF and F samples were all correctly classified. These results were similar to the specific markers approach of Model 1, acorn-fed samples being the group where the model failed. The score plot of this OPLS-DA model is shown in Fig. 5. VIP analysis with SIMCA®

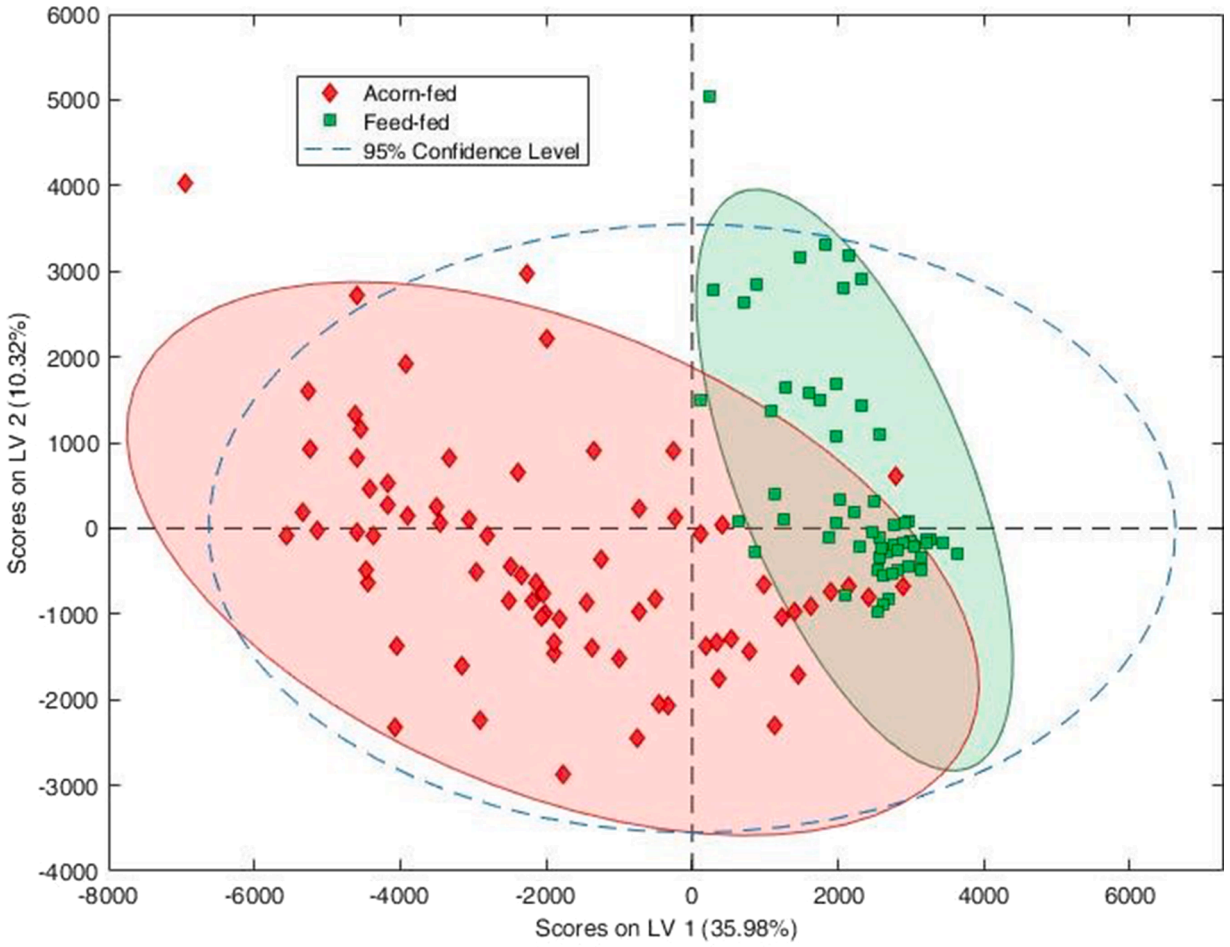

Fig. 2. PLS-DA model with fingerprint information for acorn-fed vs feed-fed classification (Model 1).

Table 4
Validation matrix for acorn-fed vs feed-fed classification (Model 1) using OPLS-DA model with specific markers information.

		Real classification	
		Acorn-fed	Feed-fed
Model prediction	Acorn-fed	13	0
	Feed-fed	1	12
	Total	14	12
	% category	92.30%	100%
	% total	96.20%	

pointed out ethyl 2-methylpropanoate, heptanal, 2,3-butanediol, propylsulfide, 2-pentanone and 2-methylbutan-1-ol as significant markers for commercial classification purpose.

These results highlight the effectiveness of GC-IMS to classify faeces samples through volatilome information according to current commercial categories and the corresponding feeding regime of Iberian pig production in Spain. Unlike Model 1, this differentiation by commercial category reflects what consumers can find on the market. A clarification should be extended here: while Spanish regulation establishes four commercial categories for Iberian products (depending on breed purity and diet), only three of them can be differentiated in terms of the animal feeding regime; within acorn-fed products, a distinction is made between those which are obtained from 100% Iberian pigs (identified with a black seal or label by the official regulation) or from animals with 50% or 75% of breed purity (identified with a red seal or label) (Ministerio de Agricultura Pesca y Alimentación, 2014). Nowadays, while the breed purity of Iberian pigs can be determined with genetic analysis, the feeding regime, in contrast, constitutes the most controversial topic for the Iberian pig industry, which is commonly discussed.

Model 2 achieved a 100% classification success for samples of groups OF and F. However, and similarly to Model 1, mistakes were observed for samples from group A: only two and one sample, respectively, were misclassified in fingerprint and marker approaches. In both, the misclassified samples from group A were classified inside the OF group, which is the most similar group in terms of animal rearing and feeding. As explained before, animals from farms of group OF, although mainly based on feed feeding, can be reared under extensive or outdoors systems and sometimes eat some natural resources. Therefore, the profile of samples of groups A and OF may be similar, which explains these classification mistakes. Otherwise, particular attention should be given to these mistakes if samples from group A were classified in group F, where animals are intensively reared under indoor systems with feed. In addition, the mistakes observed are consistent taking into account the eventual use of a fraudulent feeding strategy called “postre” (meaning dessert or snack) on some farms from group A. This consists of the use of suplementary feed as part of the animals diet during the fattening period based on acorns, which should be exclusively based on natural resources to be authentic acorn-fed. This feed strategy looks for a higher stocking rate and faster fattening, while clashing with the uniqueness of the acorn-fed commercial category defined by Spanish regulation, which only allows pigs foraging natural resources (Ministerio de Agricultura Pesca y Alimentación, 2014).

3.3. General discussion, misclassified samples, and approach selection

The results obtained in the present study show the potential of the

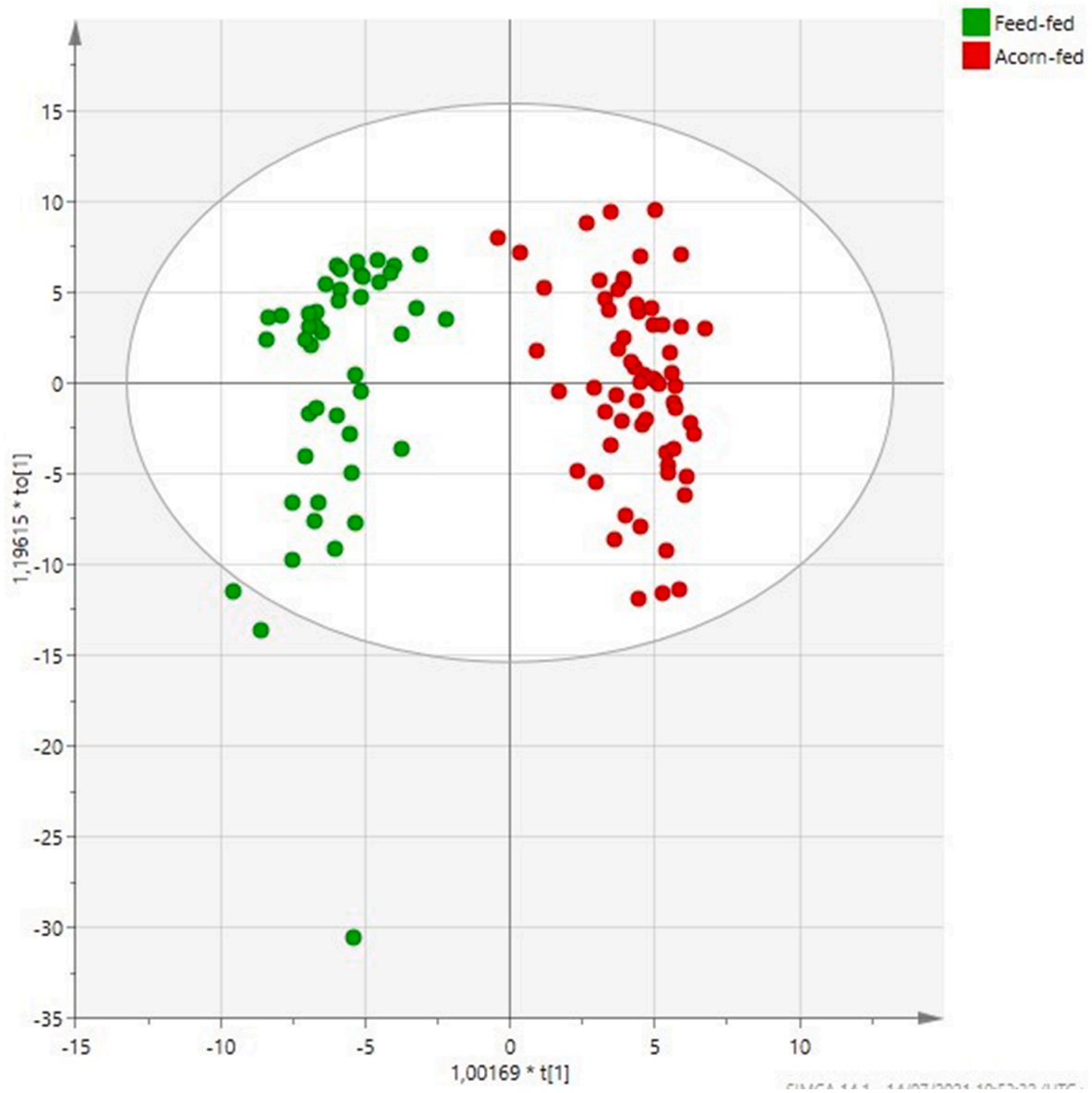

Fig. 3. OPLS-DA model with specific marker information for acorn-fed vs feed-fed classification (Model 1).

Table 5
Validation matrix for commercial category classification (Model 2) using PLS-DA model with fingerprint information.

		Real classification		
		Acorn (A)	Outdoor feed (OF)	Feed (F)
Model prediction	Acorn (A)	8	0	0
	Outdoor feed (OF)	2	8	0
	Feed (F)	0	0	8
	Total	10	8	8
	% category	80%	100%	100%
	% total	92.30%		

combination of GC and IMS to authenticate the feeding regime of the Iberian pig and the subsequent highly appreciated cured Iberian products. It could avoid the certification and selling of animals at a higher price than their feeding regime establishes because all feed-fed animals (Model 1) and animals from OF and F groups (Model 2) were correctly predicted, avoiding their classification as a superior category: acorn-fed animals (Model 1) or animals from A group (Model 2).

The production of acorn-fed Iberian pigs contributes to the preservation of the *dehesa* and its biodiversity (Bayés-García et al., 2016; Rodríguez-Estévez et al., 2012). This environmental contribution is part of the increasingly appreciated added value of cured products from acorn-fed Iberian pigs, which consumers are willing to pay for. All the above-mentioned information explains the high price that these products attain on the market, being objective of fraud (Cartín-Rojas, 2017).

GC-IMS has already been employed before to authenticate different food products with success such as olive oil, honey or even cured Iberian ham (Esteki et al., 2020, 2018; Martín-Gómez et al., 2019). This technique does not require any sample treatment and therefore reduces the final time of analysis (Arroyo-Manzanares et al., 2018). Although GC-IMS is not a popular coupling, its applications in food research have increased in recent years due to its advantages over other traditional techniques: a lower price, a portability working option and the combination of the high selectivity of GC separation with IMS good sensitivity facilitates its implementaton in agri-food laboratories (Arroyo-Manzanares et al., 2018; Hernández-Mesa et al., 2017; Karpas, 2013; Martín-Gómez et al., 2019). Besides that, the subsequent chemometric

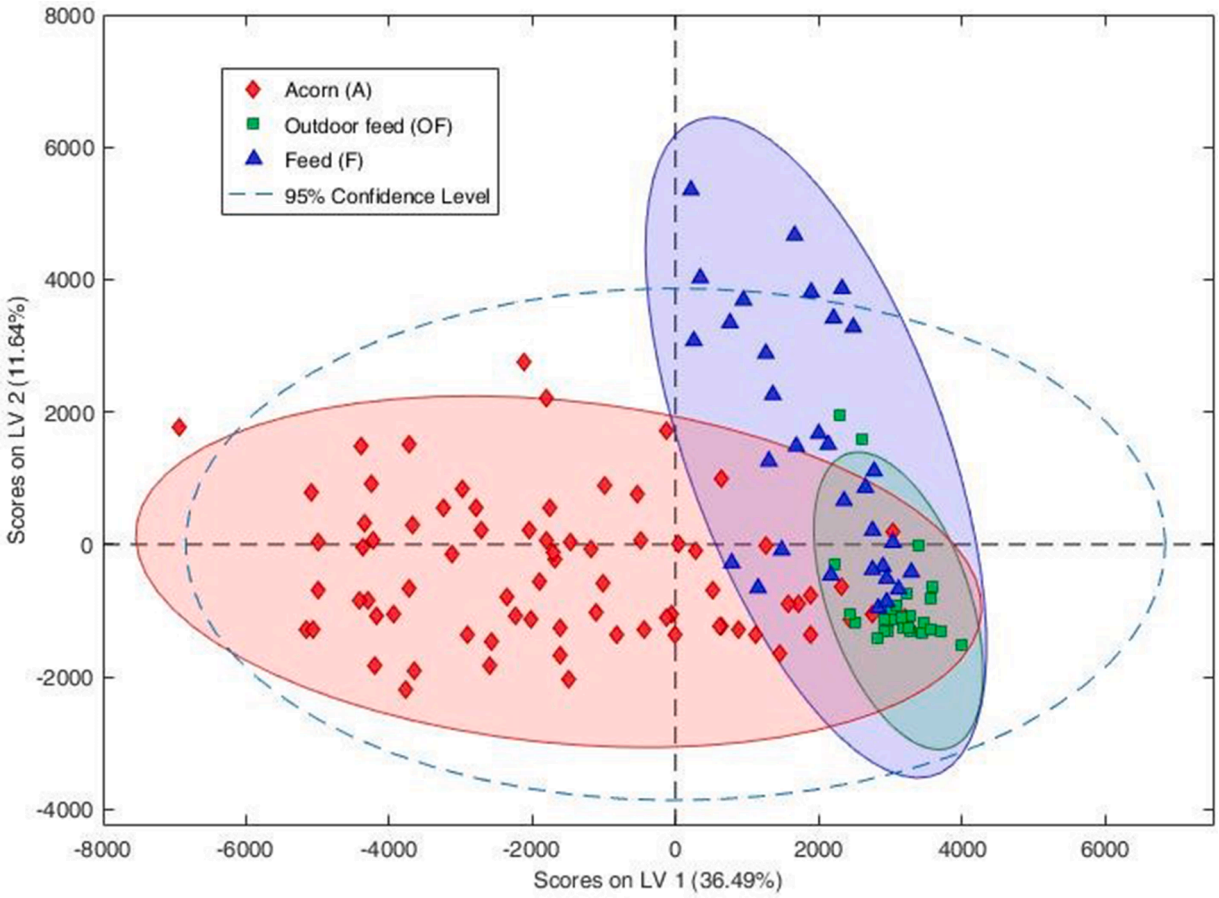

Fig. 4. PLS-DA model with fingerprint information for commercial category classification (Model 2).

Table 6
Validation matrix for commercial category classification (Model 2) using OPLS-DA model with specific markers information.

		Real classification		
		Acorn (A)	Outdoor feed (OF)	Feed (F)
Model prediction	Acorn (A)	9	0	0
	Outdoor feed (OF)	1	8	0
	Feed (F)	0	0	8
	Total	10	8	8
	% category	90%	100%	100%
	% total	96.20%		

processing of the great quantities of data generated allows multiple interesting applications. In this regard, while the majority of applications of GC-IMS for food authentication are focused on the study of final products, our study constitutes a preliminary approximation for an *in vivo* authentication of the feeding regime of Iberian pig production and it could be a promising technique to inspect organic livestock farming.

The classification mistakes detected in the present study during the model's development may be related to an uncontrolled use of supplementary feed ("postre"). Nowadays there is some controversy over this fraudulent practice because it implies a lower dependence on natural resources. It facilitates pigs handling in large areas: animals can be monitored while eating "postre" even though the rest of the day the pigs forage a long time and walk large distances to look for acorns under very low stocking rates (<1 pig/ha) (Rodríguez-Estévez et al., 2010). Furthermore, the final products are commercialised as acorn products without mention of this practice while obtaining the same economic value.

An in-depth investigation of misclassified sample origins was carried out: farmers were asked about their feeding practices to elucidate the possible explanation for the misclassification of samples from acorn-fed pigs, and they admitted sporadically giving a small amount of compound feed to attract pigs for handling ("postre"). Thus, the mistakes observed in both models can be discussed: the slight supplementation of acorn-fed animals with feed would place these samples either inside the feed-fed group (model 1) or inside group OF (model 2). Although the effectiveness of this approach has yet to be confirmed with larger sample sizes, this tool seems to be a good guarantee to prevent from classification as acorn-fed animals those which are supplemented with small amounts of feed. Misclassified samples were obtained from farms without Protected Designation of Origin inspections.

Together with the two models developed in the present study, similar percentages of classification success were obtained for the two approaches evaluated. Moreover, the same percentage of classification success was obtained for both models: while 92.3 % was obtained for the fingerprint approach, a 96.2% of samples correctly classified was observed for the marker approach. Accordingly, an extended discussion could take place about which approach should be chosen to authenticate the feeding regime of the Iberian pig. Despite the markers approach obtained better classification percentages, the fingerprint approach, although slightly less effective, seems to be the most appropiate due to the difficulty that the study of individual markers in complex matrices implies. In fact, Martín-Gómez et al. (2019) concluded that the feeding regime classification using Iberian ham was unviable using the individual marker approach. In addition, the selection of a few individual compounds to characterise and authenticate the feeding regime of Iberian pig production is considered dangerous because its control could be

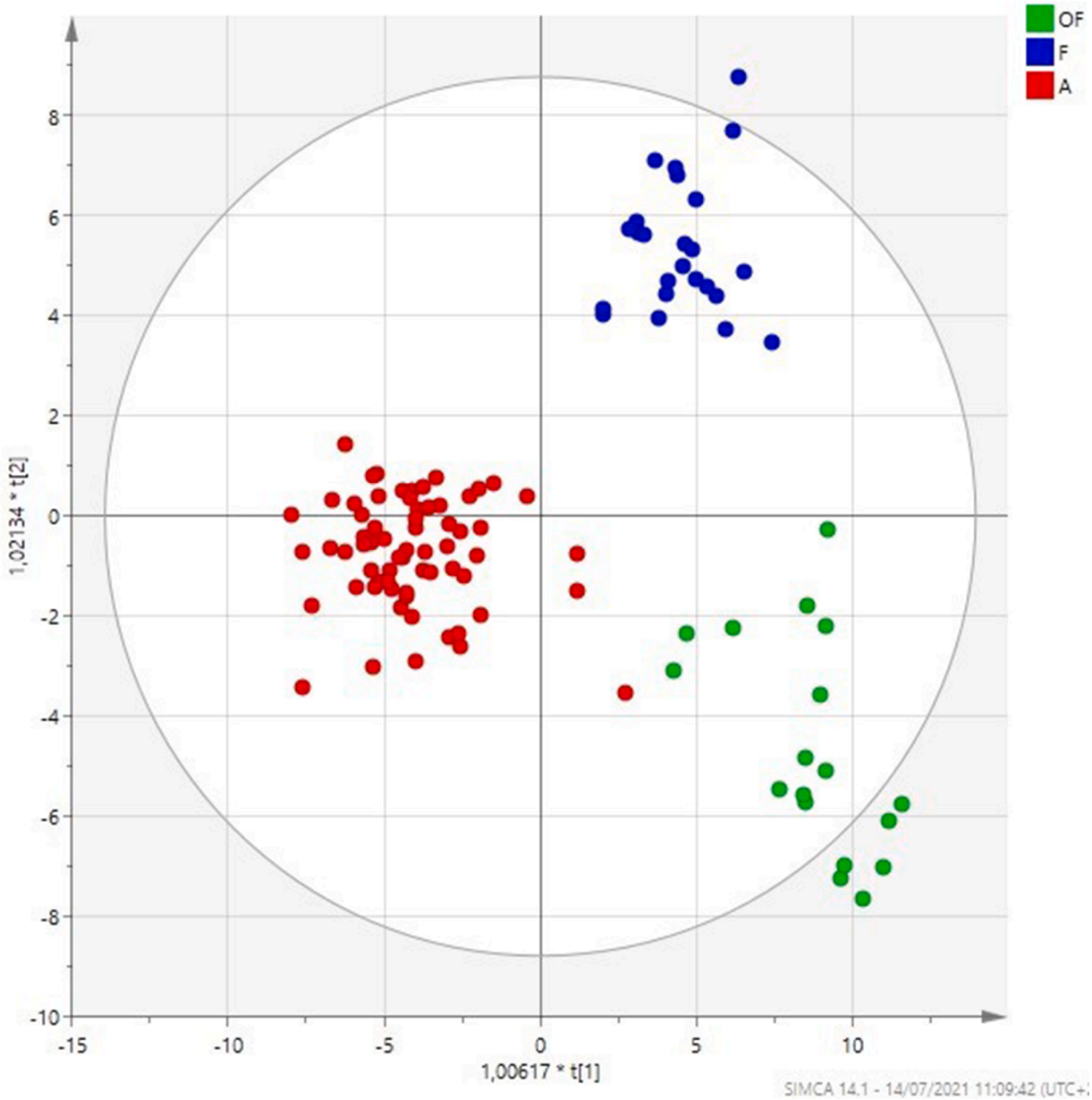

Fig. 5. OPLS-DA model with specific marker information for commercial category classification (Model 2).

avoided by modifying the composition of pig suplementary feed in order to obtain profiles similar to acorn-fed ones, eluding control and commiting fraud. Actually, in the recent past, the classification of Iberian products was attempted by using the analysis of four fatty acids according to an official test; however, the feed industry quickly learnt to imitate this profile (Alonso et al., 2008; López-Vidal et al., 2008).

In light of that situation, and as other authors have suggested before, the selection of specific markers could facilitate fraud: these compounds or their metabolic precursors could be employed to imitate higher-priced profiles through the manipulation of feed composition (Arroyo-Manzanares et al., 2018; Martín-Gómez et al., 2019). This explains the publication of only a few specific markers in the present study. This caution has been reiterated by other researchers (Arroyo-Manzanares et al., 2018) in order to prevent fraud. Hence, on the basis of the above, the entire spectral fingerprint evaluation could be considered as the most appropriate treatment for avoiding fraud.

The present study constitutes the culmination of a broad line of research carried out over 10 years in our research group to authenticate Iberian products employing GC-IMS and different samples: from subcutaneous fat sampled at the slaughterhouse (Alonso et al., 2008) to slices of dry cured Iberian ham (Arroyo-Manzanares et al., 2018) and fat from final products collected with an innovative sampling method (Martín-Gómez et al., 2019). The results presented here complement the above-mentioned approaches with a perspective unstudied to date. Thus, GC-IMS can be helpful to authenticate Iberian products and the corresponding animal feeding regime at the slaughterhouse, at point of sale to the consumers and now in animal farms. Alongside this, other techniques have been used in our research group apart from GC-IMS: infrared spectroscopy (Arce et al., 2009), GC coupled to mass spectrometry (López-Vidal et al., 2008) and raman methodologies (Martín-Gómez et al., 2021) have also shown interesting results for classification.

Although these issues were not within the scope of this study, attention should be given to breed purity and animal age. Animals included in the sampling design had both different breed purity and age, which highlights the feasibility of this methodology to study the feeding regime of Iberian pigs without the need to consider these aspects.

4. Conclusions

The development of analytical mehodologies focused on the authentication of high quality food products is considered of paramount importance for inspection and certification labours as well as to provide adequate information to consumers and clarify some confusing aspects on the market. The present study constitutes the first approximation of an *in vivo* authentication of the Iberian pig feeding regime using volatilome information from a complex and non-invasive sample such as faeces.

Given the good results achieved, the evaluation of faecal volatilome through GC-IMS could be suggested as a feasible tool to improve current certification procedures in Iberian pig products and to settle *in vivo* controls. However, there is still much work to be done to standardise GC-IMS as a solid candidate for routine analysis. The reduction of commercial categories as the Model 1 suggests that it might be helpful to clarify the market of Iberian products and facilitate consumers decisions, which often get confused due to excessive information and complex labelling. On the other hand, the model developed for the evaluation of faecal volatilome to discriminate current commercial categories of Iberian products in terms of the animal feeding regime obtained good classification results. Despite the effectiveness that GC-IMS shows for the authentication of the feeding regime of Iberian pigs, a great deal of research is still needed to obtain robust results as well as consistent conclusions. The analysis of larger number of samples is necessary to cover the highest variability possible, especially in acorn-fed groups; this would allow to obtain models able to classify quite different samples. In addition, collaboration with farms of trustwothy information about the feeding regime of outdoors fattening Iberian pigs on the *dehesa* is crucial. The combination of reliable samples and the optimisation of analytical methodologies will open new and complementary alternatives in the questioned certification of Iberian products.

Conflict of interest volatilome information

Vicente Rodríguez-Estévez (https://orcid.org/0000-0003-0148-2892), as corresponding author, certify that the authors, whose names are listed immediately below, have NO affiliations with or involvement in any organisation or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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CRediT authorship contribution statement

Pablo Rodríguez-Hernández: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **David Saavedra:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Andrés Martín-Gómez:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **M. José Cardador:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Lourdes Arce:** Conceptualization, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition. **Vicente Rodríguez-Estévez:** Conceptualization, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the present work.

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References

Alonso, R., Rodríguez-Estévez, V., Domínguez-Vidal, A., Ayora-Cañada, M.J., Arce, L., Valcárcel, M., 2008. Ion mobility spectrometry of volatile compounds from Iberian pig fat for fast feeding regime authentication. Talanta 76, 591–596. https://doi.org/10.1016/j.talanta.2008.03.052.

Amann, A., Costello, B.D.L., Miekisch, W., Schubert, J., Buszewski, B., Pleil, J., Ratcliffe, N., Risby, T., 2014. The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. J. Breath Res. 8 (3), 034001 https://doi.org/10.1088/1752-7155/8/3/034001.

Arce, L., Domínguez-Vidal, A., Rodríguez-Estévez, V., López-Vidal, S., Ayora-Cañada, M. J., Valcárcel, M., 2009. Feasibility study on the use of infrared spectroscopy for the direct authentication of Iberian pig fattening diet. Anal. Chim. Acta 636, 183–189. https://doi.org/10.1016/j.aca.2009.01.058.

Arroyo-Manzanares, N., Martín-Gómez, A., Jurado-Campos, N., Garrido-Delgado, R., Arce, C., Arce, L., 2018. Target vs spectral fingerprint data analysis of Iberian ham samples for avoiding labelling fraud using headspace – gas chromatography–ion mobility spectrometry. Food Chem. 246, 65–73. https://doi.org/10.1016/j.foodchem.2017.11.008.

Ballin, N.Z., 2010. Authentication of meat and meat products. Meat Sci. 86, 577–587. https://doi.org/10.1016/j.meatsci.2010.06.001.

Bayés-García, L., Tres, A., Vichi, S., Calvet, T., Cuevas-Diarte, M.A., Codony, R., Boatella, J., Caixach, J., Ueno, S., Guardiola, F., 2016. Authentication of Iberian dry-cured ham: new approaches by polymorphic fingerprint and ultrahigh resolution mass spectrometry. Food Control 60, 370–377. https://doi.org/10.1016/j.foodcont.2015.07.047.

Capuano, E., Boerrigter-Eenling, R., van der Veer, G., van Ruth, S.M., 2013. Analytical authentication of organic products: an overview of markers. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.5914.

Carrapiso, A.I., Bonilla, F., García, C., 2003. Effect of crossbreeding and rearing system on sensory characteristics of Iberian ham. Meat Sci. 65, 623–629. https://doi.org/10.1016/S0309-1740(02)00256-5.

Carrapiso, A.I., Noseda, B., García, C., Reina, R., Sánchez del Pulgar, J., Devlieghere, F., 2015. SIFT-MS analysis of Iberian hams from pigs reared under different conditions. Meat Sci. 104, 8–13. https://doi.org/10.1016/j.meatsci.2015.01.012.

Carrasco, J.A., Duque, J.P., 2013. Classification of Iberian pigs according to intensive feeding by chemsensor. Grasas Aceites 64, 166–172. https://doi.org/10.3989/gya.130512.

Cartín-Rojas, A., 2017. Food fraud and adulteration: a challenge for the foresight of veterinary services. Rev. Sci. Tech. Off. Int. Epiz. 36, 1015–1024. https://doi.org/10.20506/rst.36.3.2733.

Cossignani, L., Pollini, L., Blasi, F., 2019. Invited review: authentication of milk by direct and indirect analysis of triacylglycerol molecular species. J. Dairy Sci. 102, 5871–5882. https://doi.org/10.3168/jds.2019-16318.

Del Pulgar, J.S., Soukoulis, C., Biasioli, F., Cappellin, L., García, C., Gasperi, F., Granitto, P., Märk, T.D., Piasentier, E., Schuhfried, E., 2011. Rapid characterization of dry cured ham produced following different PDOs by proton transfer reaction time of flight mass spectrometry (PTR-ToF-MS). Talanta 85, 386–393. https://doi.org/10.1016/j.talanta.2011.03.077.

E Ebert, B., Halbfeld, C., M Blank, L., 2016. Exploration and exploitation of the yeast volatilome. Curr. Metabolomics 5. https://doi.org/10.2174/2213235x04666160818151119.

Esteki, M., Shahsavari, Z., Simal-Gandara, J., 2020. Gas chromatographic fingerprinting coupled to chemometrics for food authentication. Food Rev. Int. 36, 384–427. https://doi.org/10.1080/87559129.2019.1649691.

Esteki, M., Simal-Gandara, J., Shahsavari, Z., Zandbaaf, S., Dashtaki, E., Vander Heyden, Y., 2018. A review on the application of chromatographic methods, coupled to chemometrics, for food authentication. Food Control. https://doi.org/10.1016/j.foodcont.2018.06.015.

Fernández-Cabanás, V.M., Garrido-Varo, A., Olmo, J.G., Pedro, E., De Dardenne, P., 2007. Optimisation of the spectral pre-treatments used for Iberian pig fat NIR calibrations. Chemom. Intell. Lab. Syst. 87, 104–112. https://doi.org/10.1016/j.chemolab.2006.10.005.

Filipiak, W., Mochalski, P., Filipiak, A., Ager, C., Cumeras, R., E Davis, C., Agapiou, A., Unterkofler, K., Troppmair, J., 2016. A compendium of volatile organic compounds

(VOCs) released by human cell lines. Curr. Med. Chem. 23, 2112–2131. https://doi.org/10.2174/0929867323666160510122913.
Gamero-Pasadas, A., Alcaide, I.V., Rios, J.J., Constante, E.G., Vicario, I.M., León-Camacho, M., 2006. Characterization and quantification of the hydrocarbons fraction of the subcutaneous fresh fat of Iberian pig by off-line combination of high performance liquid chromatography and gas chromatography. J. Chromatogr. A 1123, 82–91. https://doi.org/10.1016/j.chroma.2006.04.081.
García-Olmo, J., Garrido-Varo, A., Pedro, E.De, 2009. Classification of real farm conditions Iberian pigs according to the feeding regime with multivariate models developed by using fatty acids composition or NIR spectral data. Grasas Aceites 60, 233–237. https://doi.org/10.3989/gya.130408.
González-Martín, I., González Pérez, C., Hernández Méndez, J., Sánchez González, C., 2001. Differentiation of dietary regimene of Iberian swine by means of isotopic analysis of carbon and sulphur in hepatic tissue. Meat Sci. 58, 25–30. https://doi.org/10.1016/S0309-1740(00)00126-1.
Hassoun, A., Måge, I., Schmidt, W.F., Temiz, H.T., Li, L., 2020. Fraud in animal origin food products : advances in emerging spectroscopic detection methods over the past five years. Foods 9 (8), 1069. https://doi.org/10.3390/foods9081069.
Hernández-Matamoros, A., González, E., García-Casco, J.M., Tejeda, J.F., 2013. Determination of neophytadiene in the subcutaneous fat of Iberian pigs from different feeding systems. Grasas Aceites 64, 173–180. https://doi.org/10.3989/gya.130612.
Hernández-Mesa, M., Escourrou, A., Monteau, F., Le Bizec, B., Dervilly-Pinel, G., 2017. Current applications and perspectives of ion mobility spectrometry to answer chemical food safety issues. TrAC Trends Anal. Chem. 94, 39–53. https://doi.org/10.1016/j.trac.2017.07.006.
Hong, E., Lee, S.Y., Jeong, J.Y., Park, J.M., Kim, B.H., Kwon, K., Chun, H.S., 2017. Modern analytical methods for the detection of food fraud and adulteration by food category. J. Sci. Food Agric. 97, 3877–3896. https://doi.org/10.1002/jsfa.8364.
Jurado-Campos, N., Martín-Gómez, A., Saavedra, D., Arce, L., 2021. Usage considerations for headspace-gas chromatography-ion mobility spectrometry as a suitable technique for qualitative analysis in a routine lab. J. Chromatogr. A 1640, 461937. https://doi.org/10.1016/j.chroma.2021.461937.
Karpas, Z., 2013. Applications of ion mobility spectrometry (IMS) in the field of foodomics. Food Res. Int. 54, 1146–1151. https://doi.org/10.1016/j.foodres.2012.11.029.
Karthikeyan, K., Muniasamy, S., SankarGanesh, D., Achiraman, S., Ramesh Saravanakumar, V., Archunan, G., 2013. Faecal chemical cues in water buffalo that facilitate estrus detection. Anim. Reprod. Sci. 138, 163–167. https://doi.org/10.1016/j.anireprosci.2013.02.017.
Lopez-Bote, C.J., 1998. Sustained utilization of the Iberian pig breed. Meat Sci. 49, S17–S27. https://doi.org/10.1016/s0309-1740(98)90036-5.
López-Vidal, S., Rodríguez-Estévez, V., Lago, S., Arce, L., Valcárcel, M., 2008. The application of GC-MS and chemometrics to categorize the feeding regime of Iberian pigs in Spain. Chromatographia 68, 593–601. https://doi.org/10.1365/s10337-008-0752-x.
Martín-Gómez, A., Arroyo-Manzanares, N., García-Nicolás, M., López-Lorente, Á.I., Cárdenas, S., López-García, I., Viñas, P., Hernández-Córdoba, M., Arce, L., 2021. Portable Raman Spectrometer as a screening tool for characterization of Iberian dry-cured ham. Foods 10, 1177. https://doi.org/10.3390/foods10061177.
Martín-Gómez, A., Arroyo-Manzanares, N., Rodríguez-Estévez, V., Arce, L., 2019. Use of a non-destructive sampling method for characterization of Iberian cured ham breed and feeding regime using GC-IMS. Meat Sci. 152, 146–154. https://doi.org/10.1016/j.meatsci.2019.02.018.
Ministerio de Agricultura Pesca y Alimentación, 2014. Disposición 879 del BOE núm. 25 de 2014. Boletín Ofical del Estado.
Pérez-Calvo, E., Wicaksono, A.N., Canet, E., Daulton, E., Ens, W., Hoeller, U., Verlhac, V., Celi, P., Covington, J.A., 2019. The measurement of volatile organic compounds in faeces of piglets as a tool to assess gastrointestinal functionality. Biosyst. Eng. 184, 122–129. https://doi.org/10.1016/j.biosystemseng.2019.06.005.
Recharla, N., Kim, K., Park, J., Jeong, J., Jeong, Y., Lee, H., Hwang, O., Ryu, J., Baek, Y., Oh, Y., Park, S., 2017. Effects of amino acid composition in pig diet on odorous compounds and microbial characteristics of swine excreta. J. Anim. Sci. Technol. 59 https://doi.org/10.1186/s40781-017-0153-5.
Rey, A.I., Daza, A., López-Carrasco, C., López-Bote, C.J., 2006. Feeding Iberian pigs with acorns and grass in either free-range or confinement affects the carcass characteristics and fatty acids and tocopherols accumulation in Longissimus dorsi muscle and backfat. Meat Sci. 73, 66–74. https://doi.org/10.1016/j.meatsci.2005.10.018.
Rey, A.I., López-Bote, C.J., 2014. Alpha-tocopherol stereoisomer analysis as discriminant method for distinguishing Iberian pig feed intake during the fattening phase. Food Chem. 142, 342–348. https://doi.org/10.1016/j.foodchem.2013.07.055.
Rodríguez-Estévez, V., García, A., Peña, F., Gómez, A.G., 2009. Foraging of Iberian fattening pigs grazing natural pasture in the dehesa. Livest. Sci. 120, 135–143. https://doi.org/10.1016/j.livsci.2008.05.006.
Rodríguez-Estévez, V., Sanchez-Rodriguez, M., Arce, C., R, A., M, J., Gustavo, A., 2012. Consumption of acorns by finishing iberian pigs and their function in the conservation of the dehesa agroecosystem. Agroforestry for Biodiversity and Ecosystem Services - Science and Practice. https://doi.org/10.5772/34877.
Rodríguez-Estévez, V., Sánchez-Rodríguez, M., García, A., Gómez-Castro, A.G., 2010. Feed conversion rate and estimated energy balance of free grazing Iberian pigs. Livest. Sci. 132, 152–156. https://doi.org/10.1016/j.livsci.2010.05.019.
Rodríguez-Hernández, P., Cardador, M.J., Arce, L., Rodríguez-Estévez, V., 2020. Analytical tools for disease diagnosis in animals via fecal volatilome. Crit. Rev. Anal. Chem. 0, 1–16. https://doi.org/10.1080/10408347.2020.1843130.
Rodríguez-Hernández, P., Rodríguez-Estévez, V., Arce, L., Gómez-Laguna, J., 2021. Application of volatilome analysis to the diagnosis of mycobacteria infection in livestock. Front. Vet. Sci. 8 https://doi.org/10.3389/fvets.2021.635155.
Santos, J.P., García, M., Aleixandre, M., Horrillo, M.C., Gutiérrez, J., Sayago, I., Fernández, M.J., Arés, L., 2004. Electronic nose for the identification of pig feeding and ripening time in Iberian hams. Meat Sci. 66, 727–732. https://doi.org/10.1016/j.meatsci.2003.07.005.
Singh, K.D., Del Miguel, G.V., Gaugg, M.T., Ibañez, A.J., Zenobi, R., Kohler, M., Frey, U., Sinues, P.M.L., 2018. Translating secondary electrospray ionization-high-resolution mass spectrometry to the clinical environment. J. Breath Res. 12 https://doi.org/10.1088/1752-7163/aa9ee3.
Sinha, R., Khot, L.R., Schroeder, B.K., Si, Y., 2017. Rapid and non–destructive detection of pectobacterium carotovorum causing soft rot in stored potatoes through volatile biomarkers sensing. Crop Prot. 93, 122–131. https://doi.org/10.1016/j.cropro.2016.11.028.
Tejero Rioseras, A., Garcia Gomez, D., Ebert, B.E., Blank, L.M., Ibáñez, A.J., Sinues, P.M.L., 2017. Comprehensive real-time analysis of the yeast volatilome. Sci. Rep. 7, 14236. https://doi.org/10.1038/s41598-017-14554-y.
Trienekens, J., Petersen, B., Wognum, N., Brinkmann, D., 2009. European Pork Chains: Diversity and Quality Challenges in Consumer-Oriented Production and Distribution. Wageningen Academic Publishers. https://doi.org/10.3920/978-90-8686-660-1.
Trivedi, D., 2012. The Application of SIMCA P+ in shotgun metabolomics analysis of ZIC®HILIC-MS spectra of human urine - experience with the shimadzu IT-T of and profiling solutions data extraction software. J. Chromatogr. Sep. Tech. https://doi.org/10.4172/2157-7064.1000145, 03.
Ventanas, S., Ventanas, J., Ruiz, J., Estévez, M., 2005. Iberian pigs for the development of high-quality cured products. Recent Res. Dev. Agric. Food Chem. 6, 1–27.Research Singpost Trivandrum, Kerala, India.
Viera-Alcaide, I., Vicarlo, I.M., Escudero-Gilete, M.L., Graciani Constante, E., León-Camacho, M., 2008. A multivariate study of the triacylglycerols composition of the subcutaneous adipose tissue of Iberian pig in relation to the fattening diet and genotype. Grasas Aceites 59, 327–336. https://doi.org/10.3989/gya.2008.v59.i4.526.