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ORIGINAL PAPER

Authentication of fattening diet of Iberian pigs according to their volatile compounds profile from raw subcutaneous fat

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Abstract The composition of volatile components of subcutaneous fat from Iberian pig has been studied. Purge and trap gas chromatography–mass spectrometry has been used. The composition of the volatile fraction of subcutaneous fat has been used for authentication purposes of different types of Iberian pig fat. Three types of this product have been considered, *montanera*, *extensive cebo* and *intensive cebo*. With classification purposes, several pattern recognition techniques have been applied. In order to find out possible tendencies in the sample distribution as well as the discriminant power of the variables, principal component analysis was applied as visualisation technique. Linear discriminant analysis (LDA) and soft independent modelling by class analogy (SIMCA) were used to obtain suitable classification models. LDA and SIMCA allowed the differentiation of three fattening diets by using the contents in 2,2,4,6,6-pentamethyl-heptane, *m*-xylene, 2,4-dimethyl-heptane, 6-*methyl*-tridecane, 1-methoxy-2-propanol, isopropyl alcohol, *o*-xylene, 3-*ethyl*-2,2-dimethyl-oxirane, 2,6-dimethyl-undecane, 3-*methyl*-3-pentanol and limonene.

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Introduction

The characterization of animal food products is a major market issue in the developed countries. Iberian pig (*Sus mediterraneus*) production system is a traditional one that has survived due to the highly demanded characteristics of the derived dry-cured products (ham), with long taste and rich nutty flavour [1, 2]. An interesting characteristic of this race is associated to the great capacity to accumulate fat under its skin and between the muscular fibres. This fat is what produces the typical white streaks that make its ham so appreciated by the consumer. The production of Iberian pig is deeply bound to the Mediterranean ecosystem. The outdoor rearing system in the Mediterranean silvopastoral system has a positive image for consumers since it is associated with an increase in animal welfare, reduced environmental impact and protection of a traditional production system [3]. The animals are reared outdoor, grazed on acorns (*Quercus* spp.) and pasture, in what is usually called *montanera* (M). Frequently, the animals are fed with formulated feed during the final fattening period. This system is called *cebo*. When pigs are fed with concentrated feed and pasture in an extensive system the fattening diet is called *extensive cebo* (EC) and *intensive cebo* (IC) when they are fed only with concentrated feed in an intensive system. The highest quality products, according to the sensory and nutritional attributes, are those derived from animals grown extensively in *montanera* and the products originated from *cebo* animals are of less quality [4]. Consequently, the Iberian pig products from

montanera fattening have a higher price and this raises the possibility of fraud.

Chemical analysis can be applied in order to differentiate the products obtained from these fattening systems. Within this realm, the study of the volatile components of meat and fat may provide useful information to characterise the products obtained from animals with different types of diet. Different factors, like the diet of the animal, have an influence in generation of volatile compounds. In this way, several studies about the composition of the volatile fraction from raw meat such as lamb [5], duck, pork and goose [6] have been carried out. Although volatile compounds have been widely studied as tracers of animal feeding systems [7], only a few researches have focused on volatile compounds present in raw subcutaneous fat, being these done in lamb [8]. In the case of Iberian pig, there is not any study about the influence of the type of feeding in volatile fraction from subcutaneous fat tissue. So, more information about the volatile profile of Iberian pig fat is needed to construct a generic traceability strategy based on the analysis of fat and to relate them with the different fattening system. Volatile flavour profile of meat samples can be studied using several techniques such as steam distillation-extraction, dynamic headspace extraction on Tenax and solid-phase microextraction [9]. After the concentration step gas chromatography [10] can be applied to determine the volatile components.

In this work, a study of the volatile fraction of raw subcutaneous fat from Iberian pig has been carried out. The volatile composition of fat samples obtained from animals grown in *montanera*, *extensive cebo* and *intensive cebo* has been determined by purge and trap gas chromatography −mass spectrometry (GC–MS). The determined compounds have been used as chemical descriptors to differentiate these three fattening diets. With this aim, several pattern recognition techniques have been applied. In order to find out possible tendencies in the sample distribution as well as the discriminant power of the variables, principal component analysis (PCA) was applied as visualisation technique in order to detect patterns in the data. Linear discriminant analysis (LDA) and soft independent modelling by class analogy (SIMCA) were used to obtain suitable classification models.

Materials and methods

Chemicals and reagents

2-Ethylhexyl-2-propenoate [103-11-7], carbon disulfide [75-15-0], 1-ethyl-3-*methyl*-cyclopentanoate [3726-47-4], isopropanol [67-63-0], 1-methoxy-2-propanol [107-98-2], cyclobutylamine [2516-34-9], toluene [108-88-3], hexanal [66-25-1], 2,3-dimethyl-2-butanol [594-60-5], 2-*methyl*-2-pentanol [590-36-3], 3-*methyl*-3-pentanol [77-74-7], *p*-xylene [106-42-3], *m*-xylene [108-38-3], *o*-xylene [95-47-6], heptanal [111-71-7], limonene [5989-27-5] and 1-pentanol [71-41-0] were obtained from Sigma Aldrich Fluka (Steinheim, Germany). Standard solutions were prepared using fully deodorised edible oil as matrix. Concentrations were in the range 0.1–5.0 μg/g.

Fat samples

A total of 75 samples of fat from castrated 14-month-old male Iberian pure pig were analysed. Thirty-five corresponding to animals with a fattening diet based exclusively on acorn (*Quercus ilex,Quercus suber* and *Quercus faginea*) and pasture for 90 days prior to slaughter. This type of diet is usually called M. Thirteen corresponding to animals fed with commercial feed and pasture in an extensive system, called EC and 27 corresponding to animals fed with commercial feed in an intensive system, called IC. Samples were kindly provided by the designations of origin (DO) Los Pedroches and Jamón de Huelva, located in the southwest of Spain. The animals were classified in these three different groups according to the field notes taken by the veterinary inspector of the DO during the final fattening period. Raw samples were obtained following the method proposed by the Spanish regulation [11]. They were stored at −18 °C until analysis was carried out. Before analysis, fat was minced and homogenised in order to increase the interface between the sample and the striping gas during the concentration step.

Apparatus and methods

Purge and trap GC–MS

The volatile compounds were isolated from three grammes of sample prepared as it is indicated above by dynamic headspace. A Purge and Trap Concentrator apparatus Tekmar Velocity XPT (Thousand Oaks, CA, USA) with a Tenax trap was used. The purge conditions were as follows: sample temperature, 45 °C; Tenax trap temperature, 35 °C; purge gas flow, 350 mL min^{-1} of nitrogen; purge time: 14 min. After the purge period, the volatile compounds were desorbed, holding the Tenax trap at 225 °C for 1 min, and sent through the transfer line, kept at 150 °C, into the gas chromatograph injector. A Varian 3,800 gas chromatograph coupled to a Saturn 2,000 ion trap mass spectrometer (Varian, Palo Alto, CA, USA) equipped with a 1,079 injector and a Supelcowax-10 (SUPELCO, Bellefonte, PA, USA) fused silica capillary column (60 m long × 0.25 mm i.d. × 0.25 μm film thickness) was used. Hydrogen was

used as carrier at 1.6 mL min^{-1} in constant flow mode. The oven temperature was held at 40 °C for 14 min and programmed to rise 1 °C min^{-1} to a temperature of 91 °C, then at 10 °C min^{-1} to a temperature of 201 °C, and then to rise at 5 °C min^{-1} to a final temperature of 220 °C where it was held for 20 min. Split injection mode was used with a ratio of 1:5. The injector temperature was kept at 250 °C. Mass spectrometer was operated in full-scan mode from 20 to 300 amu at one scan per second. Ion source and transfer line temperatures were 200 and 290 °C, respectively. The electron energy was 70 eV, unit resolution and a emission current of 250 μA were fixed. Dwell time and inter-channel delay were 0.08 and 0.02 s, respectively. Varian MS Workstation version 6.3 software was used for data acquisition and processing of the results. The compounds present in the volatile fraction of the fat samples were identified by computer matching of their mass spectra against the NIST and Wiley libraries and verified by standard addition. Toluene was used as a reference to calculate the relative retention time, due to it appears in all samples with high intensity at a mean retention time of 7.80 min. Peak area was used as analytical signal. The quantification of individual volatile compounds was carried out by evaluating the corresponding relative percentage according to the area normalisation method, assuming an equal relative response factor for any species.

Chemometrics

The volatile compounds identified were considered as chemical descriptors. A data matrix whose rows are the samples and columns are the variables were built. Each element of this matrix x_{ij} corresponds to the content of the volatile compound j for the sample i. Several pattern recognition techniques have been applied. In order to find out possible tendencies in the sample distribution as well as the discriminant power of the variables, PCA was applied as visualisation technique. LDA and SIMCA were used to obtain suitable classification models. The data were autoscaled before the chemometric analysis. Statistica 8.0 (StatSoft, 2007) was used for PCA and LDA calculations. SIMCA was applied with SIMCA-P (Umetrics, 2001).

Results and discussion

Purge and trap GC–MS analysis

The volatile components of the samples were separated using a high polarity column, and the conditions of the purge and trap system and GC–MS were previously described [10]. Under the conditions used in the purge step no degradation of the matrix sample was observed. Repeatability was checked and values expressed as relative standard deviation ranged between 13.1% and 22.8%. Recoveries for the analytes with available standard varied between 92% and 115%. Figure 1 shows a chromatogram of the volatile fraction of Iberian pig raw subcutaneous fat. Twenty-seven volatile compounds have been identified. The relative retention time, molecular ion and base peak of the corresponding peaks are included in Table 1. Several aliphatic hydrocarbons are present in the volatile fraction of Iberian pig raw subcutaneous fat. Branched hydrocarbons such as 2,2,4,6,6-pentamethyl-heptane, 2,2,4,4-tetramethyl-octane, 2,6,7-trimethyl-decane, 6-*methyl*-tridecane, 2,4-dimethyl-heptane, 2,6-dimethyl-undecane have been detected, being these two last compounds previously described by several authors in the Iberian dry-cured ham [10, 12]. The aromatic hydrocarbons toluene, *o*-xylene, *m*-xylene, *p*-xylene, 1,3,5-trimethyl-benzene were also present. Some authors have cited the presence of these compounds in the volatile fraction of pig fat [13, 14]. The terpenic hydrocarbon limonene [13] is also present. Alcohols, such as isopropanol, 2-*methyl*-2-pentanol and 1-pentanol have also been detected in the samples, and their presence in Iberian dry-cured ham was previously mentioned by some authors [12, 13, 15]. The contribution to flavor of the aldehydes is very important. Four of them, hexanal, heptanal, octanal and nonanal have been identified in raw subcutaneous fat samples for the first time in the present work. However, they have been previously described in dry-cured ham [13]. Other compounds like 2-ethylhexyl-2-propenoate, carbon disulfide, 3-ethyl-2,2-dimethyl-oxirane and cyclobutylamine are also present. Only one of them, carbon disulfide, was previously described in Iberian dry-cured ham literature [15].

Table 2 shows the median, minimum and maximum percentages of the compounds detected in the volatile fraction of the studied samples corresponding to the three types of diet considered. It can be seen that compounds such as 2,4-dimethyl-heptane, 2,2,4,6,6-pentamethyl-heptane and 2-ethylhexyl 2-propenoate are the most abundant compounds, with median values of 43.88%, 11.44% and 13.20%, respectively. Other major components, with median values higher than 1%, were carbon disulfide, 3-ethyl-2,2-dimethyl-oxirane, 1-methoxy-2-propanol, cyclobutylamine, 2,6,7-trimethyl-decane, toluene and hexanal. The content in 6-*methyl*-tridecane was also higher than 1% for *montanera* samples. The same occurs for 1-ethyl-3-*methyl*-cyclopentane and 2,2,4,4-tetramethyl-octane for *extensive* and *intensive cebo*, respectively. Compounds such as 1-ethyl-3-*methyl*-cyclopentane, isopropanol, 2,2,4,4-tetramethyl-octane, 2,6-dimethyl-undecane, 6-*methyl*-tridecane, *p*-xylene, *o*-xylene, *m*-xylene, heptanal, limonene and octanal were present with median values higher than 0.1% and lower than 1%. Low percentages

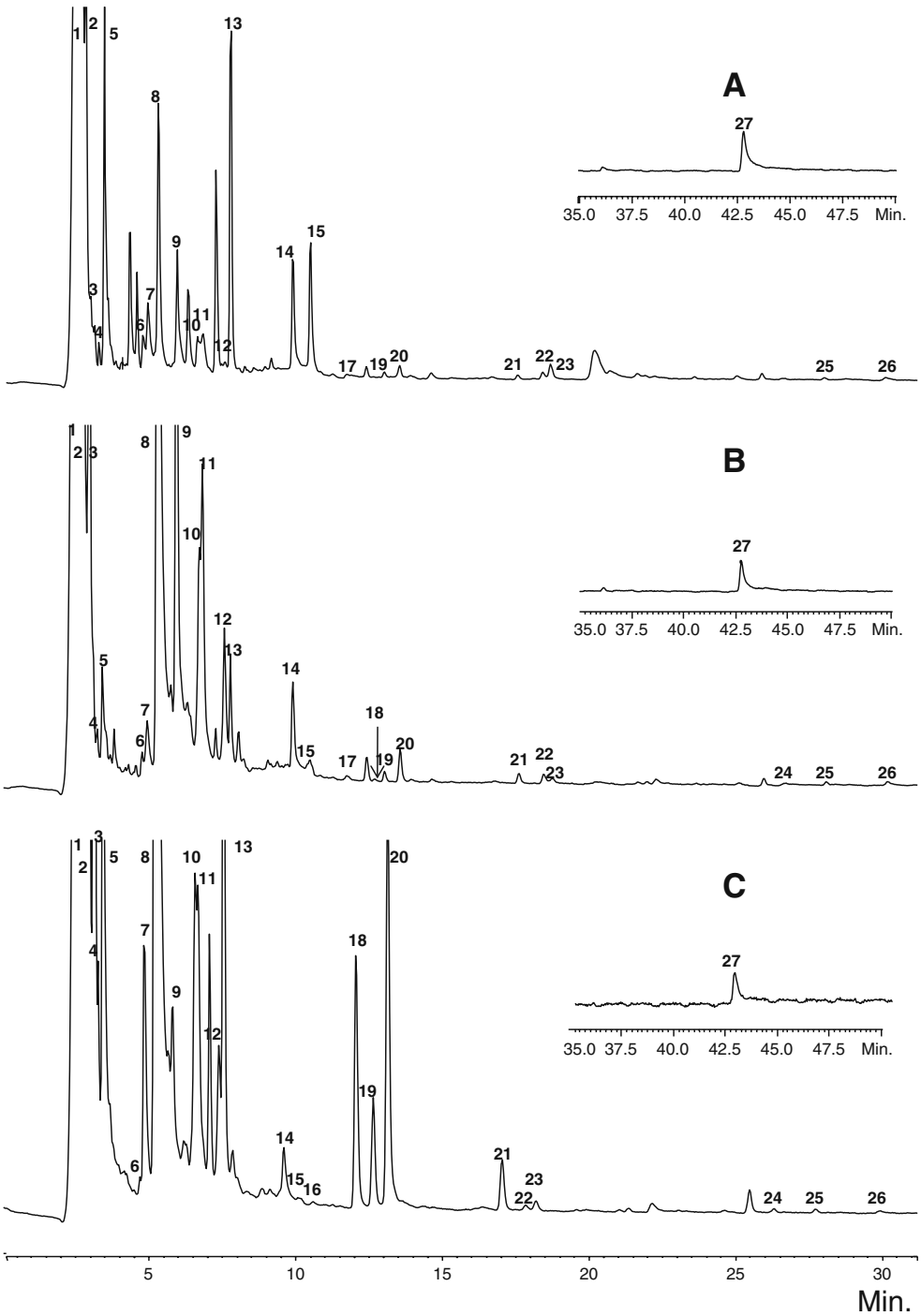

Fig. 1 GC-Ion-Trap–MS Chromatogram from 0.0 to 32.0 min in full-scan mode of the volatile compounds profile of subcutaneous fat of Iberian pig samples: *A montanera*; *B intensive cebo* and *C extensive cebo* fattening diet. See Table 1 for peaks identification

were found for trimethyl-bencene, 1-pentanol, 2-*methyl*-2-pentanol, 3-*methyl*-3-pentanol and 2,3-dimethyl-2-butanol.

Classification of fat samples

Principal component analysis

In order to find out possible tendencies in the sample distribution as well as the discriminant power of the variables PCA was applied as visualisation technique [16]. The two first PCs were extracted explaining the 33.2% and 16.8% of the total variance, respectively. Figure 2 shows the biplot in the plane of the two first PCs. As can be seen the most contributing variables to PC1 were 2-ethylhexyl 2-propenoate, 3-ethyl-2,2-dimethyl-oxirane, isopropanol, 2,2,4,6,6-pentamethyl-heptane, 2,6-dimethyl-undecane, 6-*methyl*-tridecane, toluene, limonene and 2,4-dimethyl-heptane. PC2 highest loadings were found for xylene

Table 1 Volatile compounds identified in Iberian pig raw subcutaneous fat

Peak	Compound	Identification	T_{RR}	Base peak	M^+
1	2,4-Dimethyl-heptane	L	0.35	43	128
2	2-Ethylhexyl-2-propenoate	S	0.36	55	184
3	Carbon disulfide	S	0.38	76	76
4	1-Ethyl-3-*methyl*-cyclopentane	S	0.41	55	112
5	3-Ethyl-2,2-dimethyl-oxirane	L	0.45	59	100
6	Isopropanol	S	0.62	45	60
7	1-Methoxy-2-propanol	S	0.63	45	90
8	2,2,4,6,6-Pentamethyl-heptane	L	0.68	57	170
9	Cyclobutylamine	S	0.77	43	71
10	2,2,4,4-Tetramethyl-octane	L	0.86	57	170
11	2,6,7-Trimethyl-decane	L	0.87	57	184
12	2,6-Dimethyl-undecane	L	0.97	57	184
13	Toluene	S	1.00	91	92
14	Hexanal	S	1.27	44	100
15	6-*Methyl*-tridecane	L	1.33	57	198
16	2,3-Dimethyl-2-butanol	S	1.42	59	102
17	2-*Methyl*-2-pentanol	S	1.50	59	102
18	3-*Methyl*-3-pentanol	S	1.62	73	102
19	*p*-Xylene	S	1.67	91	106
20	*m*-Xylene	S	1.74	91	106
21	*o*-Xylene	S	2.26	91	106
22	Heptanal	S	2.36	44	114
23	Limonene	S	2.41	68	136
24	1-Pentanol	S	3.41	42	88
25	1,3,5-Trimethyl-benzene	L	3.58	105	120
26	Octanal	S	3.86	41	128
27	Nonanal	S	5.48	57	142

L library, *S* standard, T_{RR} relative retention time, M^+ molecular ion

isomers. Considering the scores for PC1 *montanera* appears separately from *intensive* and *extensive cebo* samples. These two classes present different values of PC2 scores. These results seem to indicate that a linear approach, such as LDA or SIMCA, can be used to construct a classification model in order to differentiate among the three considered fattening diets.

Linear discriminant analysis

Forward stepwise LDA was applied with the aim of constructing a classification model as well as reducing the number of variables [17]. The datamatrix was divided in two subsets. A training set, containing the 66% of the samples, was used to construct the model. This was then applied to the test set, including 33% of the samples, to obtain the classification performance of the constructed model. Forward stepwise analysis retains the most discriminant variables: 2,2,4,6,6-pentamethyl-heptane, *m*-xylene, 2,4-dimethyl-heptane, 6-*methyl*-tridecane, 1-methoxy-2-propanol, isopropanol, *o*-xylene, 3-ethyl-2,2-dimethyl-oxirane, 2,6-dimethyl-undecane, 3-*methyl*-3-pentanol and limonene. After applying this model to the test set 100% classification performance was obtained for the three considered classes. Figure 3 shows the sample distribution in the space of the two obtained discriminant functions. Samples of *montanera*, *extensive cebo* and *intensive cebo* appear completely separated.

Soft independent modelling by class analogy

As LDA is a hard modelling technique, samples are forced to be classified as one of the considered classes. In order to make this classification more reliable, a soft modelling technique, such as SIMCA, should be used. By applying SIMCA, samples can be or cannot be classified in one of the considered groups, making the constructed model more flexible. SIMCA determined the number of principal components needed to describe the structure of each class in the training set. The boundaries of each class are obtained and objects are included in a class if they fall into the *n*-dimensional box limited by these boundaries [18].

Table 2 Composition (%) of volatile fraction of Iberian pig fat

Compound	*Intensive cebo*	*Extensive cebo*	*Montanera*
2,4-Dimethyl-heptane	45.06 (30.17–66.12)	70.28 (48.16–89.41)	34.15 (21.55–62.70)
2-Ethylhexyl 2-propenoate	6.51 (0.84–18.35)	3.06 (0.12–10.54)	16.66 (12.24–30.07)
Carbon disulfide	1.66 (ND–8.35)	ND	4.28 (ND–6.25)
1-Ethyl-3-*methyl*-cyclopentane	0.24 (ND–1.60)	1.74 (0.97–3.04)	ND (ND–4.90)
3-Ethyl-2,2-dimethyl-oxirane	0.88 (0.09–2.40)	2.72 (1.11–6.02)	9.28 (3.27–20.57)
Isopropanol	0.11 (ND–0.26)	0.02 (ND–0.15)	0.81 (ND–2.59)
1-Methoxy-2-propanol	0.62 (0.25–3.15)	0.17 (0.06–1.73)	2.26 (0.51–13.26)
2,2,4,6,6-Pentamethyl-heptane	31.01 (15.73–50.51)	13.61 (5.26–21.35)	4.30 (2.13–8.79)
Cyclobutylamine	1.51 (0.31–7.39)	0.1 (0.03–0.21)	4.01 (0.92–9.35)
2,2,4,4-Tetramethyl-octane	1.39 (0.23–2.54)	0.52 (0.25–1.05)	0.55 (ND–8.43)
2,6,7-Trimethyl-decane	2.11 (1.21–3.82)	0.76 (0.36–1.44)	1.96 (ND–10.97)
2,6-Dimethyl-undecane	0.85 (ND–1.37)	0.31 (0.10–0.50)	ND (ND–0.41)
Toluene	0.61 (0.26–1.83)	1.41 (0.72–2.21)	6.51 (3.22–12.24)
Hexanal	0.88 (0.46–4.44)	0.18 (0.08–0.55)	2.05 (0.72–6.28)
6-*Methyl*-tridecane	0.07 (ND–0.29)	0.02 (ND–0.08)	2.85 (1.19–4.69)
2,3-Dimethyl-2-butanol	0.02 (ND–0.06)	ND (ND–0.01)	ND (ND–0.23)
2-*Methyl*-2-pentanol	0.04 (ND–0.19)	ND (ND–0.02)	ND (ND–0.18)
3-*Methyl*-3-pentanol	0.06 (ND–0.32)	ND	ND (ND–0.09)
p-Xylene	0.05 (ND–0.09)	0.34 (0.17–0.65)	0.15 (0.09–0.43)
m-Xylene	0.17 (0.09–0.30)	1.73 (0.87–3.10)	0.33 (0.13–0.96)
o-Xylene	0.05 (ND–0.11)	0.19 (0.09–0.41)	0.17 (ND–0.36)
Heptanal	0.13 (0.04–0.30)	0.02 (ND–0.07)	0.29 (0.11–1.65)
Limonene	0.11 (ND–0.73)	0.02 (ND–0.07)	0.4 (0.17–0.73)
1-Pentanol	0.03 (ND–0.15)	0.02 (ND–0.03)	ND (ND–0.13)
Trimethyl-benzene	0.03 (ND–0.07)	0.01 (ND–0.02)	ND (ND–0.18)
Octanal	0.07 (ND–0.34)	0.01 (ND–0.03)	0.13 (0.04–1.66)
Nonanal	0.32 (ND–0.79)	0.08 (ND–0.29)	0.88 (0.17–19.4)

Median (minimum–maximum)

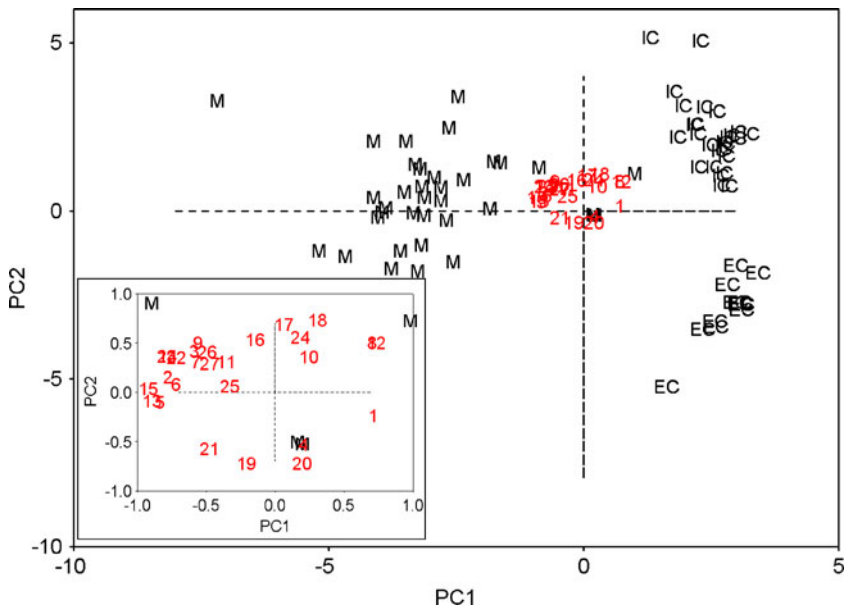

Fig. 2 Biplot in the plane of the two first PCs. *M montanera*, *IC intensive cebo*, *EC extensive cebo*. Variables are numbered according to the peak number in Table 1. *Left bottom square* includes a zoom for a better visualisation of the variables

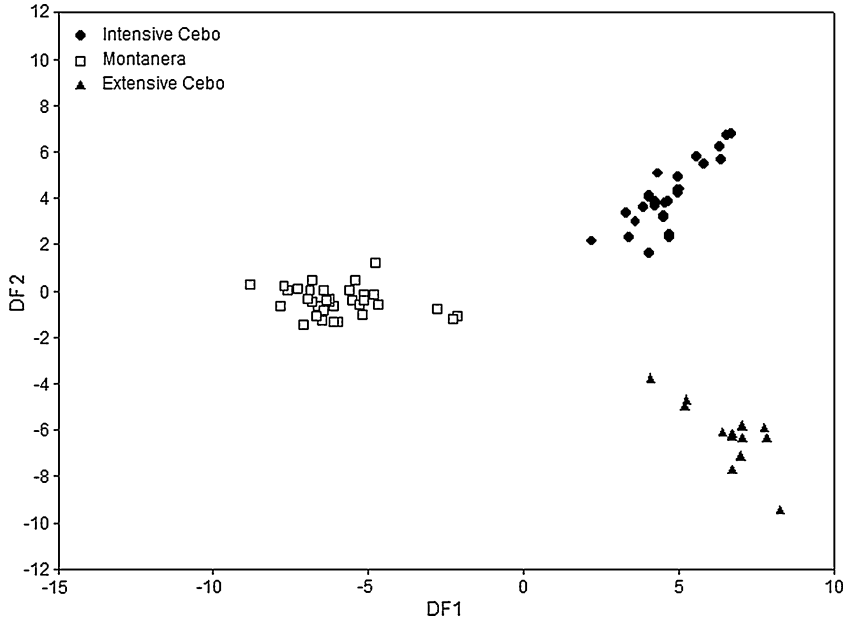

Fig. 3 Sample distribution in the plane of the obtained DFs

Using the variables selected by the forward LDA and the same division of the datamatrix, a SIMCA model was obtained. The Kaiser criterion [19] was used to determine the number of components to be retained for each class. Taking into account that the data are autoscaled, each observed variable contributes one unit of variance to the total variance in the data set. The Kaiser criterion retains components with eigenvalues greater than 1, because these components account for a greater amount of variance than one observed variable. In this case, the four first PCs were extracted for *montanera* and *intensive cebo* and the two first for *extensive cebo* classes. When the model was applied to the test set, a 100% of classification performance was achieved. A useful tool to visualise SIMCA results is the Coomans plot. The distance of cases from the model for a considered class is plotted against that from the model for the other considered class. Figure 4 shows the Coomans plot for *montanera* and *intensive cebo*. As can be seen, all samples from these classes appear in the zone of their corresponding classes being correctly assigned. Additionally, all *extensive cebo* cases are depicted out of the critical distance lines.

Conclusions

Taking into account the results of LDA and SIMCA, it can be concluded that samples of Iberian pig fat can be differentiated according to the three considered fattening diet by using the contents in 2,2,4,6,6-pentamethyl-heptane, *m*-xylene, 2,4-dimethyl-heptane, 6-*methyl*-tridecane, 1-methoxy-2-propanol, isopropanol, *o*-xylene, 3-ethyl-2,2-dimethyl-oxirane, 2,6-dimethyl-undecane, 3-*methyl*-3-pentanol and limonene. These models could be used to establish a quality control system of the products before

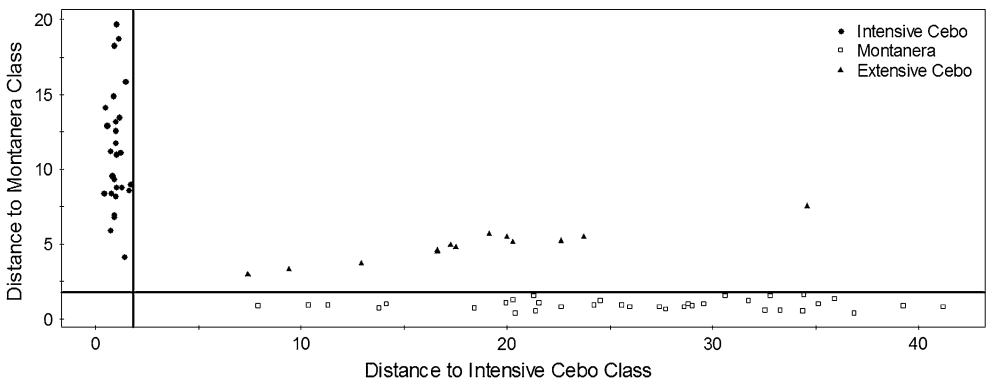

Fig. 4 Coomans plot

the elaboration process is finished, but these trends should be confirmed with a higher number of samples.

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