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Different fattening systems of Iberian pig according to 1-alkene hydrocarbon content in the subcutaneous fat

By **I. Viera-Alcaide[1], M. Narváez-Rivas[1], I.M. Vicario[2], E. Graciani-Constante[1],** and **M. León-Camacho[1,*]**

[1] Instituto de la Grasa (C.S.I.C.), Food Characterization and Quality Department. Seville, Spain.
[2] Department of Nutrition and Food Science. Faculty of Pharmacy. University of Seville. Spain.
* Corresponding author: e-mail: mleon@cica.es

RESUMEN

Diferentes sistemas de cebado del cerdo Ibérico según el contenido de 1-alqueno en la grasa subcutánea.

El contenido de n-alquenos de la grasa subcutánea de 755 muestras procedentes de cerdos ibéricos machos, se ha determinado mediante combinación off-line de los métodos HPLC y GC. Las muestras correspondían a tres grupos según el tipo de alimentación en el período final de engorde ("*Montanera*", alimentados con bellota y pasto; "*Recebo*", alimentados con bellota, pasto y pienso; y "*Cebo*", alimentados con pienso y pasto). Usando los n-alquenos como descriptores químicos, se aplicaron técnicas de análisis multivariante para diferenciar entre los tres tipos de alimentación de cerdo ibérico. Se encontró que las variables más diferenciadores fueron n-$C_{16:1}$, n-$C_{18:1}$, n-$C_{22:1}$ y n-$C_{24:1}$. Sin embargo, el total de las muestras no están clasificadas, mejorando el nivel de clasificación cuando se eliminan las muestras correspondientes a animales de "*Recebo*". Para el "*Cebo*", se encontró una relación entre los niveles de n-$C_{14:1}$, n-$C_{16:1}$ y n-$C_{18:1}$ y el período de sacrificio, siendo los niveles de estos muy bajos cuando los animales, no eran de cría en extensivo y no tenían pasto en su dieta de engorde.

PALABRAS CLAVE: Cerdo ibérico – Cromatografía gaseosa – Grasa subcutánea – 1-alquenos – Sistemas de engorde.

SUMMARY

Different fattening systems of Iberian pigs according to the 1-alkene hydrocarbon content in the subcutaneous fat.

The n-Alkene content in samples of subcutaneous fat corresponding to 755 castrated male Iberian pigs has been determined by an off-line combination of HPLC and GC method. The samples corresponded to three groups based on the type of feeding during the finish fattening period ("*Montanera*", fed on acorns and pasture; "*Recebo*", fed on acorns, feed and pasture; and "*Cebo*", fed on feed and pasture). By using the n-alkenes as chemical descriptors, multivariate statistical techniques were applied to differentiate between the three fattening diet types for Iberian pigs. The most differentiating variables were n-$C_{16:1}$, n-$C_{18:1}$, n-$C_{22:1}$ and n-$C_{24:1}$. However, a clear classification of the samples was not achieved. The level of classification was improved when the data corresponding to the animals fed with the "*Recebo*" fattening diet was removed from the analysis. A relationship between n-$C_{14:1}$, n-$C_{16:1}$ and n-$C_{18:1}$ levels and the slaughter period was found to be very low for the animals fed with the "*Cebo*" fattening diet when the animals had not been closely managed and pasture had not been included in their fattening diet.

KEY-WORDS: Fattening systems – Gas chromatography – Iberian pig – 1-alkenes – Subcutaneous fat.

1. INTRODUCTION

Nowadays consumers demand high quality products with particular sensory or health properties. The products made from Iberian pig meat comply with these requirements since they have a much appreciated organoleptic taste and they can be considered healthy from their fat composition point of view (high in oleic acid) García Rebollo *et al*. (1998). The Iberian pig *(Sus Mediterraneus*) production systems is one of the few traditional ones (together with certified Parma ham production) which has survived due to the highly demanded characteristics of the derived dry-cured products, with a lasting taste and rich nutty flavor (Andersen *et al*., 2005; Bosi *et al*., 2000). Besides, the outdoor rearing in the Mediterranean silvopastoral system (called "*La Dehesa*"), 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 (López Bote, 1998; Rey *et al*., 2006; Bénédicte and Guillard, 2005). The animals are reared outdoors, grazing on acorns (Quercus spp) and pasture, in what is usually called "*Montanera*". The key problem of this system is that the availability of resources in "La Dehesa" is limited and sometimes it is necessary to complete feeding during the final fattening period with formulated feeds which are usually called "*Recebo*". On other farms, the animals are fed formulated feed during the final fattening period in order to reach the adequate slaughter weight; this system is called "*Cebo*". Classification of the animals into one of these groups has an economic and industrial effect, since the best quality, highly priced dry-cured hams are related to "*Montanera*" animals followed by "*Recebo"* and "C*ebo"*. At the moment, the fatty acid analysis of the subcutaneous fat by gas chromatography (GC) is

an unsatisfactory criterion for classifying the animals according to the three feeding system (B.O.E., 2007). Other analytical parameters are being explored as alternatives to fatty acids to establish the feeding system of the animals previous to slaughter. In this sense triacylglycerol analysis has been proposed as an alternative to fatty acids (Viera-Alcaide *et al*., 2007; Viera-Alcaide *et al*., 2008) which improves classification results. The hydrocarbon fraction has also been explored. Hydrocarbons are components of vegetable waxes which have been used for authentication purposes (León-Camacho and Morales, 2000). Their presence in animal´ tissue has been related to the diet consumed by the animals during the fattening period (Bandurski and Nagi, 1975). The traditional feeding of Iberian pigs is based on acorns and pasture. In the hydrocarbon profile of acorns medium chain-alkanes (C_{29} and C_{27}) predominate and comprise about 80% of total hydrocarbons detected, while other n-alkanes account for less than 10% (León-Camacho *et al*. 2004)). In the fresh fat of Iberian pigs (Tejeda *et al*., 1999; Tejeda *et al*., 2001a; Tejeda *et al*., 2001b; Gamero-Pasadas *et al*., 2006) and in intramuscular fat of dry-cured hams (Petrón *et al*., 2004; Petrón *et al*., 2005; Petrón *et al*., 2006), several authors have described the linear saturated hydrocarbons, from n-C_{14} up to n-C_{29} except n-C_{23} and n-C_{28} n-alkanes, n-alkenes from n-C_{12} to n-C_{32}, and branched hydrocarbons. Short chain hydrocarbons under n-C_{12} have not yet been identified. In relation to branch hydrocarbons, some of them remain unidentified, probably due to analytical problems. To improve the isolation of the unsaponifiable fraction of lipids, thin layer chromatography (TLC) Petrón *et al*. (2006) and liquid chromatography (LC) Tejeda *et al*. (2001b) are being replaced by HPLC methods (León-Camacho and Morales, 2000). Recently a new HPLC-GC off line method that allows isolation and quantification of the hydrocarbon fraction was reported by Gamero-Pasadas *et al*. (2006) who also described a new diterpenic hydrocarbon, ent-Kaurene, for the first time in the subcutaneous fresh fat of the Iberian pig.

Although some authors have reported that the differences in linear hydrocarbons are insufficient at the moment to discriminate hams from Iberian pigs fattened on different diets, they have reported diet-related differences in some branched hydrocarbons Petrón *et al*. (2006).

The aim of this work was to study the n-alkene hydrocarbon profile of the subcutanous fat of Iberian pigs reared extensively on the three types of fattening diets: "*Montanera*" (*M*), "*Cebo*" (*C*) and "*Recebo*" (*R*). The effect of the rearing system on the hydrocarbon composition was also studied. The final objective was to explore the feasibility of using the n-alkene hydrocarbon profile for classification purposes of samples corresponding to different fattening diets.

2. MATERIALS AND METHODS

2.1. Animals and samples

The samples analyzed in this study were representative of 50640 castrated male Iberian pigs reared extensively under the guidelines of the Designation of Origin "Jamón de Huelva". Data were collected during three consecutives campaigns (2002 to 2005). One group of animals (23966 animals) was fed on acorns and pasture, this system is usually called "*Montanera*". Other group (13925 animals) was fed with feed and pasture, usually called "*Cebo*" and the last group (12749 animals) was fed with acorns, feed and pasture, usually called "*Recebo*". Animals were assigned to the different groups according to the field notes taken by the veterinary inspector of the Designation of Origin.

Samples

A total of 755 samples of Iberian pig fat were analyzed (Table 1). Raw samples were obtained by the official method (BOE, 2004) and the representative sample of the lot was obtained as described elsewhere Viera-Alcaide *et al*. (2008). The unsaponifiable fraction was extracted following the procedure described in Gamero-Pasadas *et al*. (2006). Briefly, 1 mL of the Standard solution of n-eicosane was added to 20g of fat. The mixture was saponified for one hour with 75 mL of 10% ethanolic potassium hydroxide. The solution was transferred to a 500-mL decanting funnel, 100 mL distilled water were added and the mixture was extracted twice with tree 100-mL portions of hexane. The hexane extracts were combined in another funnel and were washed several times with 100-mL portions of a mixture of ethanol-water (1:1), until the wash was at a neutral pH. The hexane solution was dried over anhydrous sodium sulphate and

Table 1
Total animals and samples analyzed from different campaigns

Campaign	"*Montanera*"		"*Recebo*"		"*Cebo*"	
	samples	animals	Samples	animals	Samples	animals
2002-2003	174	10572	71	5087	89	6171
2003-2004	119	7740	61	3515	48	3646
2004-2005	83	5654	45	4147	65	4108

evaporated to dryness in a rotary evaporator at 30°C under reduced pressure.

2.2. *HPLC separation*

The HPLC system consisted of an Agilent (Palo Alto, CA, USA) 1100 liquid chromatograph, with a quaternary pump, a Rheodyne (Cotati, CA, USA) injection valve (300 μL loop), a Peltier furnace and a refractive index detector. A Valco Instruments Co. Inc. (Bandera, TX, USA) valve model VT-E90 was installed at the output of the detector for the recovery of the hydrocarbon fraction. A Chemical station HP was used for controlling and monitoring the system. The separation was performed in a 250 × 4 mm particle size 5 μm Lichrospher Si 60 Merck (Darmstadt, Germany) column. The temperature of the column and the detector were held, respectively, at 30 and 35°C. The mobile phase was n-hexane/ethyl acetate 85/15 (v/v). The flow rate was supported at 1 mL min^{-1} for 30 min.

The complete unsaponifiable fraction was dissolved in approximately 300 μL of mobile phase, and the solution was injected into the HPLC system. The fraction that eluted from minute 1.0 to 4.50 was recovered through the VALCO valve and concentrated in the rotary evaporator at 30°C under reduced pressure to 0.15 mL and analyzed by gas chromatography.

2.3. *GC Analysis*

The hydrocarbon fraction was analyzed as described elsewhere Gamero-Pasadas *et al*. (2006). A VARIAN (Palo Alto, CA, USA) 3800 gas chromatograph equipped with a split/splitless injector and a flame ionization detector; a Capillary column (30 m × 0,22 mm i.d.) coated with a 0,12 μm film of a Teknokroma TRB-ESTEROL stationary phase and a VARIAN 8100 automatic injector were used. The temperature program was as follows: initial temperature at 114°C, 4°C min^{-1} to 310°C, followed by an isothermal period of 11 min at the latter temperature. The injector and detector were held at 280 and 320°C respectively. Hydrogen was used as carrier gas at a constant head pressure of 10 psi, and a split ratio of 1:40 was used. Air and hydrogen with flow rates of 300 and 30 mL min^{-1}, respectively, were used for the detector, which had an auxiliary flow of 30 mL min^{-1} of nitrogen. Table 2 shows the 1-alkenes of the subcutaneous fat of the Iberian pigs used in this study and their retention times.

Each component in the chromatogram was quantified using n-eicosane as internal standard. The response factor relative to n-alkanes and n-alkenes was close the unit.

2.4. Statistical analysis

For classification purposes, ANOVA-MANOVA, Principal Component (PCA) and Linear Discriminant Analysis (LDA) were applied to the hydrocarbon composition to infer differences between groups according to the fattening diet. The Bonferroni test was applied for *Post-hoc* comparisons. For LDA the criterion used for the feature selection of variables included in the model was the forward stepwise approach. The variables were included in the model according to their discriminating power based on the Wilks´λ statistic test (StatSoft, 2002). The multivariate statistical analysis was performed using the software Statistica® v 6.0 (Statsoft Inc., 2001)

3. RESULTS AND DISCUSSION

3.1. n-Alquene profile of adipose tissue

The contents of single n-alkene determined (mean and standard deviation) are shown in Table 2, together with the corresponding retention times. Twenty-one n-alquenes, including even (ECNE) and odd (OCNE) carbon number n-alquenes were identified in the samples subjected to the HPLC_GC method. The n-alkene profile was characterized by a predominance of ECNE (7.96 ±4.10 mg·kg^{-1}) among which the main components were those from $C_{14:1}$ to $C_{18:1}$ (5.73±3.38 mg·kg^{-1}), in accordance with previously published data for the intramuscular fat of Iberian dry-cured ham (Petrón *et al*., 2004). On the contrary, odd carbon number alquenes (OCNE) were found at low levels (0.73±0.28 mg·kg^{-1}). Up to now, OCNE (from $C_{13:1}$ to $C_{31:1}$) and the very short chain hydrocarbon $C_{12:1}$ had not been described in the subcutaneous fat of Iberian pigs, probably due to the low levels in which they are found (below 0.20 mg·kg^{-1})

It has been reported that the feeding system has no significant effect on the hydrocarbon profile of Iberian ham intramuscular lipid Petrón *et al*. (2004). However, we found significant differences in most of the n-alquenes analyzed when the three feeding systems were considered as separate groups (Table 2). In general, the mean values obtained for n-alquenes were higher for the animals fed on *Montanera*, followed by *Recebo* and, finally by *Cebo* animals. This is the case of ECNE, for which the animals fed on *Montanera* presented values significantly higher ($p < 0.05$) than *Recebo* and *Cebo*. The main ECNEs presented significant differences in relation to the fattening diet. $C_{14:1}$ content was significantly ($p < 0.05$) higher for *Montanera* and *Recebo* pigs than for *Cebo,* while $C_{16:1}$ and $C_{18:1}$ presented significantly higher values for *Montanera* than for Recebo and Cebo. However, for OCNE, the highest values were found for the animals fed on *Recebo* followed by *Cebo* and *Montanera*. Similar results have been obtained by other authors for the intramuscular fat of dry-cured hams (Petrón *et al*., 2004; Petrón *et al*., 2005).

3.2. Principal components analysis

In order to view the grouping trend of the variables according to the type of fattening diet, an

Table 2
Retention time (R_t), mean (ppm) and standard Deviation (S.D.), of 1-alkenes fraction of Iberian pig subcutaneous fat

n-alkenes	R_t (min)	All Groups n=717	*Montanera* n= 365	*Recebo* n=173	*Cebo* n=179
n-$C_{12:1}$	4.14	0.20±0.35**	0.26±0.42[a]	0.19±0.30[a]	0.09±.12[b]
n-$C_{13:1}$	5.55	0.01±0.01**	0.00±0.01[a]	0.01±0.02[b]	0.01±0.02[a]
n-$C_{14:1}$	7.49	1.66±1.23**	1.81±1.20[a]	1.62±1.32[a]	1.39±1.13[b]
n-$C_{15:1}$	9.95	0.05±0.05**	0.04±0.02[a]	0.05±0.04[b]	0.08±0.08[c]
n-$C_{16:1}$	12.29	2.29±1.46**	2.52±1.47[a]	2.13±1.38[b]	1.99±1.45[b]
n-$C_{17:1}$	15.12	0.07±0.09**	0.05±0.08[a]	0.09±0.09[b]	0.10±0.11[b]
n-$C_{18:1}$	17.58	1.78±1.23**	1.96±1.28[a]	1.62±1.08[b]	1.57±1.20[b]
n-$C_{19:1}$	20.35	0.05±0.05	0.05±.004	0.06±0.06	0.06±0.05
n-$C_{20:1}$	22.71	0.40±0.54**	0.36±0.52[a]	0.57±0.67[b]	0.31±0.40[a]
n-$C_{21:1}$	25.29	0.04±0.03	0.04±0.03	0.03±0.02	0.04±0.02
n-$C_{22:1}$	27.49	0.72±0.51**	0.81±0.52[a]	0.63±0.48[b]	0.64±0.49[b]
n-$C_{23:1}$	29.88	0.05±0.04	0.05±0.04	0.05±0.03	0.05±0.04
n-$C_{24:1}$	31.93	0.38±0.27	0.41±0.28[a]	0.33±0.26[b]	0.36±0.26[a]
n-$C_{25:1}$	34.13	0.09±0.05**	0.07±0.03[a]	0.11±.06[b]	0.09±0.04[c]
n-$C_{26:1}$	36.04	0.21±0.17*	0.23±0.18[a]	0.18±0.16[b]	0.21±0.15[a]
n-$C_{27:1}$	38.08	0.10±0.04**	0.10±0.03[a]	0.12±.04[b]	0.09±0.03[c]
n-$C_{28:1}$	39.89	0.18±0.19*	0.16±.018[a]	0.21±0.22[b]	0.19±0.17[a]
n-$C_{29:1}$	41.75	0.16±0.06**	0.17±.006[a]	0.17±0.07[a]	0.12±0.03[b]
n-$C_{30:1}$	43.43	0.13±0.13	0.13±.013	0.14±0.14	0.14±0.11
n-$C_{31:1}$	45.19	0.12±0.05	0.11±.005	0.12±0.06	0.12±0.05
Σn-C_{even}		7.96±4.10**	8.65±3.54[a]	7.63±4.73[b]	6.89±4.26[c]
Σn-C_{odd}		0.73±0.28**	0.69±0.22[a]	0.81±0.35[b]	0.75±0.29[a]
Σn-$C_{14:1}$ $C_{16:1}$ $C_{18:1}$		5.73±3.38**	6.29±2.94[a]	5.37±3.69[b]	4.95±3.71[c]

** $p < 0.01$; *$p < 0.05$; means within the same row which have a different superscript are significantly different $p < 0.05$.

analysis of principal components was applied. A varimax rotation was done and 4 factors with weights higher than 1 were extracted. The total variance explained was 68.0% when the analyses included the 21 n-alkenes determined. When only OCNE were included the variance explained was higher (72.9% of the total variance) and the highest was obtained by ECNA (95.0% of the total variance). PC1 and PC2 explain up to 76.41% of the total variance, with 45.93% explained by PC1 and 30.48% by PC2. To visualize trends in the data, the scores for samples and loadings for variables were represented in the space of the two principal components (PCs) obtained by PCA Meloun *et al*. (1992). Figure 1A shows the loading of the variables in this space. As can be seen, the most contributing variable (avoiding correlation among them) are: n-$C_{24:1}$, n-$C_{22:1}$ (correlated with n-$C_{26:1}$), n-$C_{18:1}$ (correlated with n-$C_{28:1}$) and n-$C_{16:1}$ (correlated with n-$C_{30:1}$). Accordingly, the scores plot obtained by selecting the variables most contributing to PC1 (n-$C_{16:1}$, n-$C_{18:1}$, n-$C_{22:1}$ and n-$C_{24:1}$) is depicted in Figure 1B, and it can be observed that a good separation between groups was not achieved.

When the PCA was conducted in the same conditions described above but excluding the *Recebo* samples, the 4 factors extracted explained 100.0% of the total variance. The hydrocarbons which had a major positive contribution to the PC1 were the same as in the previous analysis. PC1 and PC2 explained up to 99.05% of the total variance, with 86.36 % explained by PC1 and 12.69% by PC2, but the separation between groups was not improved.

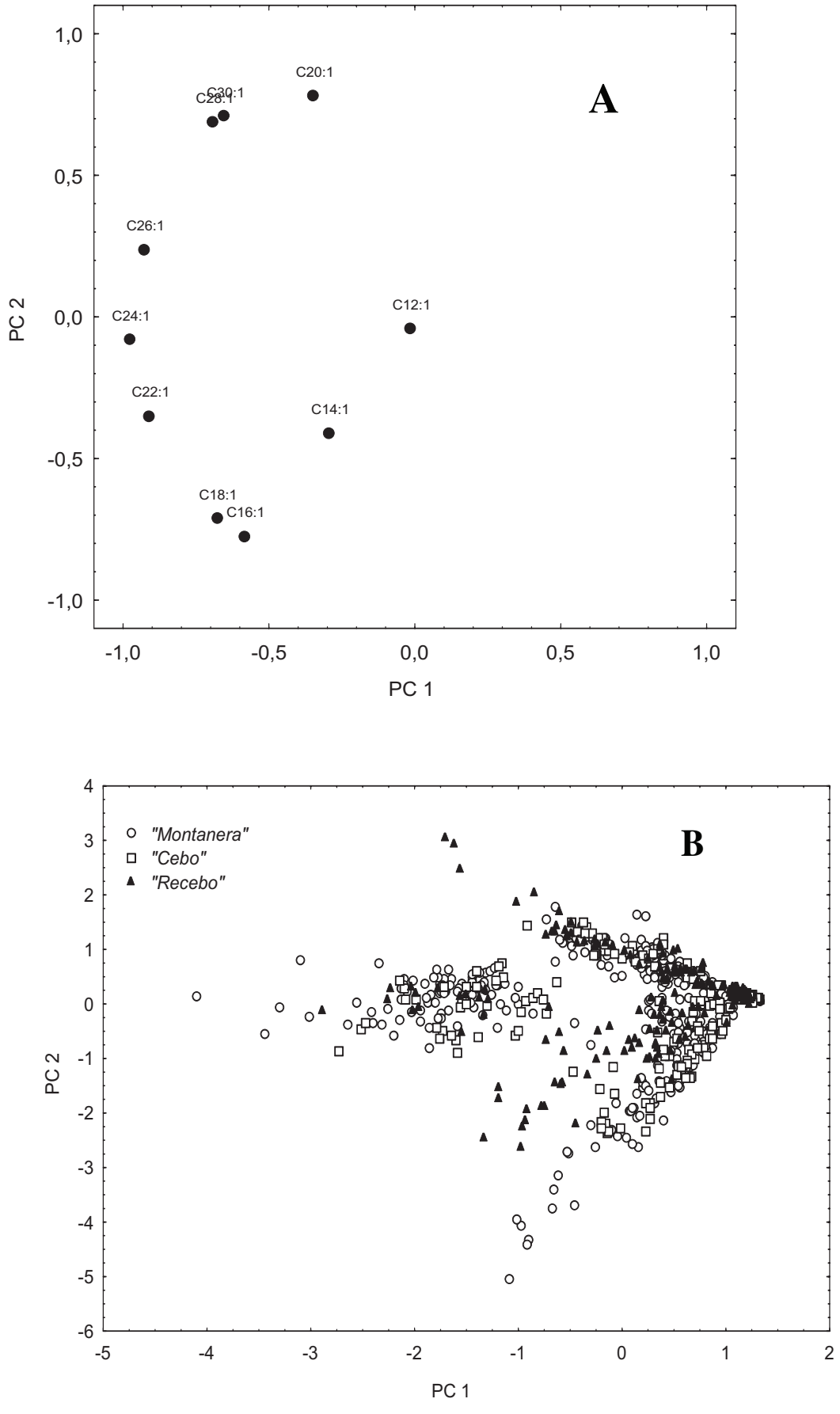

Figure 1
(**A**) Projection of the hydrocarbon variables and (**B**) the Montanera (M) and Cebo (C) and Recebo (R) samples onto the space define by PC1 and PC2.

3.3. Discriminant Analysis

To achieve a better separation of the groups according to fattening diets a linear discriminant analysis (LDA) was carried out. The 21 n-alquenes were included as variables considering "a priori" equal probability for a sample to be in one group independently of the group size. A tolerance of 0.001 was set to eliminate the variables that provided redundant information with those already included in the model. The hydrocarbons included in the model were 17. The $C_{15:1}$ hydrocarbon with a higher F value (F = 32.7) offered the best discrimination among the three groups. As can be observed in Table 3, the classification of the samples was not good, with a 73.64% of total results. The highest percentage predicted of correct classification was obtained for "*Montanera*" samples (81.09%), while the "*Recebo*" and "*Cebo*" samples were misclassified. Figure 2A shows the case discrimination, grouped by fattening diet, according to the first two canonical variables or square roots obtained from the classification functions.

In the same way that it was done with previous variables studied, an alternative classification was made considering only two distinct groups of fattening diet: "*Cebo*" and "*Montanera*". In this case, the hydrocarbons included in the model were 16. The $C_{29:1}$ hydrocarbon provided the best discrimination between the two groups. As can be observed in Table 4, the classification of the samples was better than in the previous case, in which the three fattening diets were taken into consideraton. A higher percentage of correct classifications was obtained.

Figure 2B shows the case discrimination, grouped by fattening diet, according to the first canonical variable or square roots obtained from the classification functions for the two types of fattening diets. In this figure, a significant overlap between the two groups can be observed.

Table 3
Classification matrix of the cases. Observed classifications in rows and predicted classifications in columns, for three fattening diet types

	% correct classification	M $p = 0.33333$	C $p = 0.33333$	R $p = 0.33333$
M	81.09	296	28	41
C	76.53	22	137	20
R	54.91	51	27	95
Total samples	73.64	369	192	156

Table 4
Classification matrix of the cases. Observed classifications in rows and predicted classifications in columns, for the two fattening diet types

	% correct classification	*"Montanera"* $p = 0.50000$	*"Cebo"* $p = 0.50000$
M	91.23	333	32
C	84.92	27	152
Total samples	89.15	360	184

3.4. Influence of the rearing system on hydrocarbon levels

The level of hydrocarbons present in the subcutaneous fat of the animals has been related to the fattening diet since these compounds are depositted in the animals' tissues without any modification (Bandurski and Nagi, 1975). Previous studies have addressed this point, discovering higher levels of some branch hydrocarbon in Iberian pig subcutaneous fat samples corresponding to animals fed extensively on *Montanera* for a long period of time (Tejeda *et al*., 2001; Petrón *et al*., 2006). This is an advisable result since pasture is the main source for these compounds and in extensive dietary systems the period that the animals remain eating pasture is variable depending on the available resources. In dry-cured meat, this relationship has also been reported for some branch hydrocarbons. We have explored the levels of hydrocarbons in relation to the season of the year when the animals were sacrificed (Figure 3). For "*Cebo*" animals, which are slaughtered throughout the year a decrease in the levels of the most important OCNA (sum of 1-tetradecene, 1-hexadecene and 1-octadecene) can be observed during the dry seasons, when pasture is scarce or unavailable . This can be explained by the fact that "*Cebo*" animals have been reared outdoors. It can be observed that the hydrocarbon values do not exceed 2 mg kg^{-1} of fat during summer and early autumn, while higher values are obtained in winter and spring.

4. CONCLUSIONS

According to the results obtained in this study, the n-alkene profile of subcutaneous adipose tissue of the Iberian pig is affected by the fattening diet system.

However, n-alquene is not a good parameter to discriminate among animals fed extensively on M, C and R. However for animals reared extensively on C diets, a relationship between the OCNE (n-$C_{14:1}$, n-$C_{16:1}$ y n-$C_{18:1}$) level and the season of the year when the animals are slaughtered has been found. The lowest levels (<0.2 mg·kg^{-1}) were found in animals reared intensively, when pasture was not included in their fattening diet.

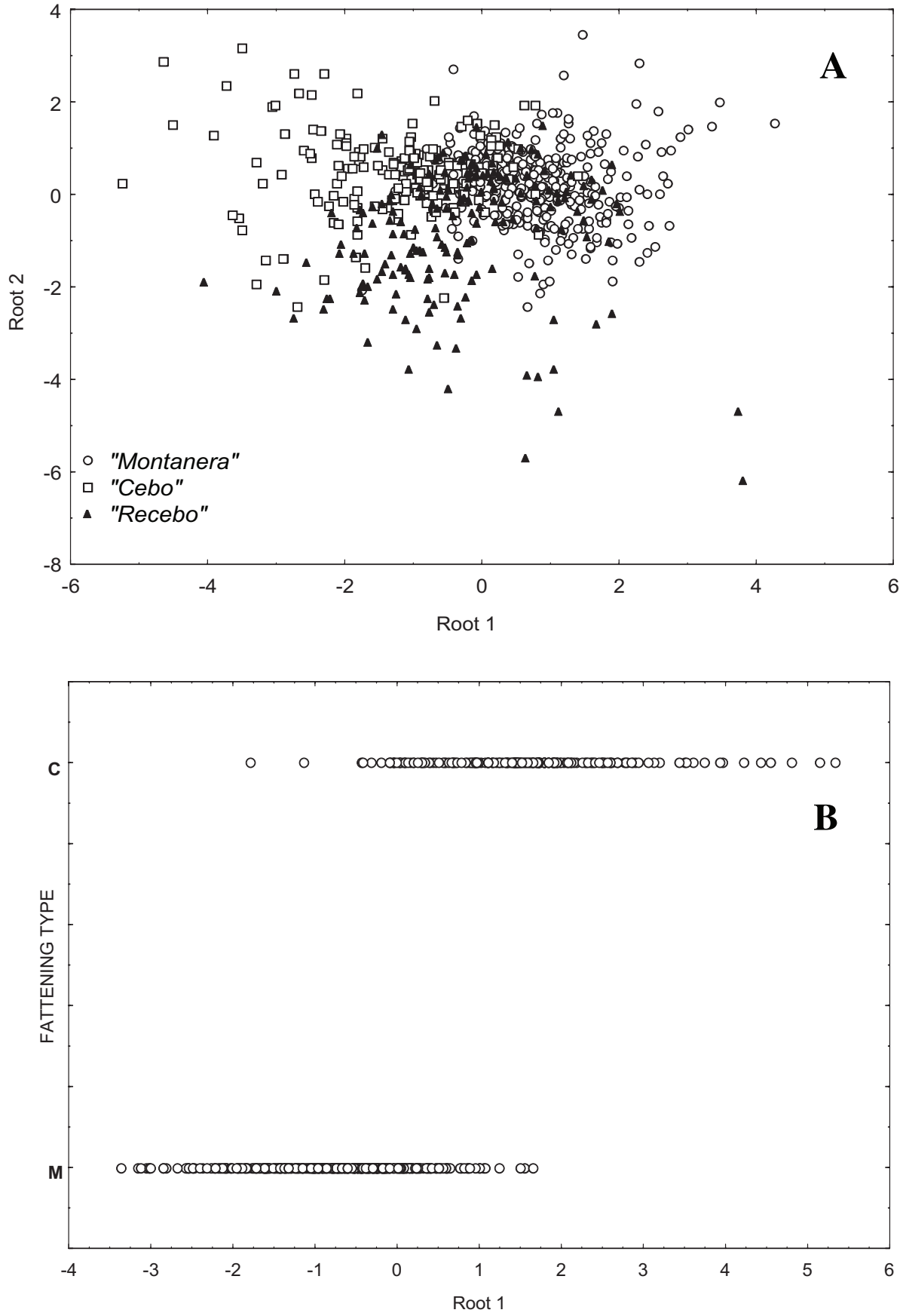

Figure 2
A: Scatterplot of the canonical scores corresponding to "*Montanera*", "*Recebo*" and "*Cebo*". **B:** Scatterplot of the canonical scores corresponding to "*Montanera*" and "*Cebo*".

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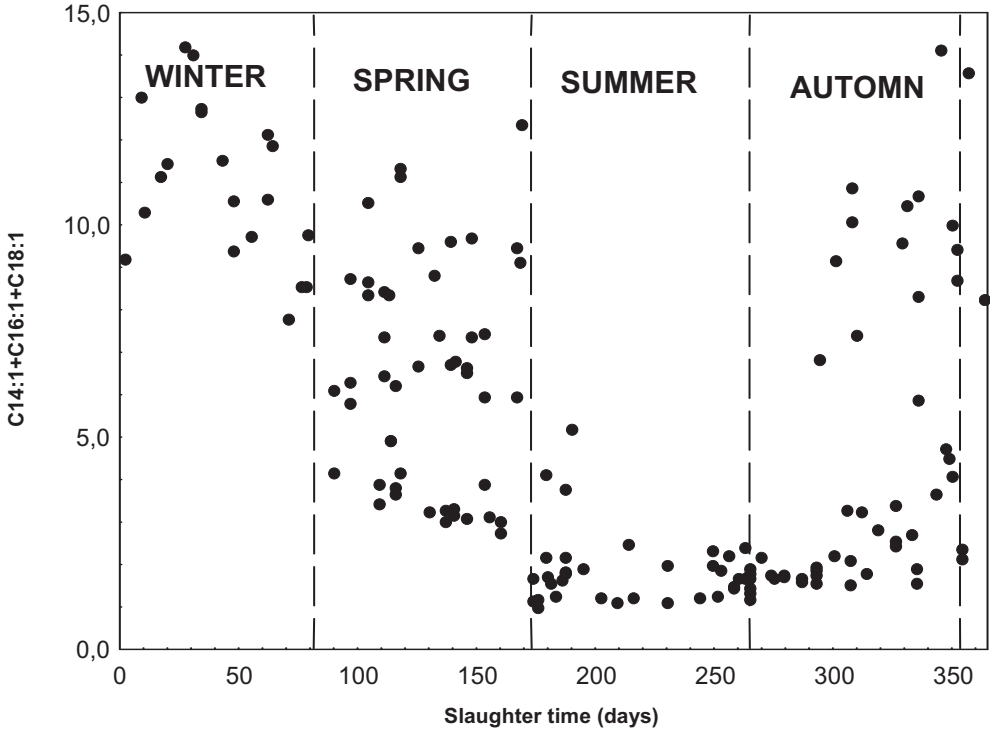

Figure 3
Even chain hydrocarbons levels (n-$C_{14:1}$ + n-$C_{16:1}$ + n-$C_{18:1}$) in mg kg^{-1} of fat *versus* slaughter period.

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