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Subcutaneous Fat Triacylglycerols Profile from Iberian Pigs as a Tool To Differentiate between Intensive and Extensive Fattening Systems

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ABSTRACT: Triacylglycerols of subcutaneous fat of Iberian pigs reared on two different feeding systems, extensive and intensive, have been determined by gas chromatography with a flame ionization detector. Analyses were performed on a column coated with a bonded stationary phase (50% phenyl−50% methylpolysiloxane) with hydrogen as the carrier gas. Lipids were extracted by melting the subcutaneous fat in a microwave oven and then filtering and dissolving in hexane. A total amount of 1995 samples from several campaigns were considered. Palmitoyl-stearyl-oleoyl glycerol and palmitoyl-dioleoyl glycerol were the most abundant triacylglycerols found in the samples. A study on the discriminating power of the triacylglycerols to differentiate samples according to the pig feeding system was performed. By using the triacylglycerols as chemical descriptors, principal component analysis, linear discriminant analysis, and soft independent modeling of class analogy were applied. Dioleoyl-linoleoyl glycerol and oleoyl-dilinoleoyl glycerol were the most discriminating variables. Variable−variable plots of these two glycerols allow separation of the samples according to their content.

KEYWORDS: *Iberian pig, subcutaneous fat, triacylglycerols, gas chromatography, pattern recognition*

INTRODUCTION

Iberian dry-cured products, like ham, are meat products manufactured according to traditional methods in the southwest regions of Spain. Iberian pig products have an extraordinary acceptance by consumers, and their demand has increased in the last years due to the culinary qualities and the system of animal outdoors rearing, which increases the animal welfare, reduces environmental impact, and protects the ecosystem.[1,2] Therefore, the factor that determines the prices in the market is the fattening diet of the animals. Traditionally, animals are often fed during the final fattening period in an extensive regime, although the use of formulated feeds in an intensive regimen has increased in recent years. The type of diet is one of the main factors that determine the composition of Iberian pig adipose tissue. Fatty acids, triacylglycerols,[3−5] hydrocarbons,[6−8] and volatile compounds[9,10] profiles are related to the type of diet and can be used as chemical descriptors to differentiate between Iberian pig products elaborated with animals fed with a different diet. The analysis of triacylglycerols shows the advantage of being faster than other methods, such as the determination of fatty acids, hydrocarbons, and volatile compounds, which require a more or less long time for the derivatization or recovery of those compounds. However, the triacylglycerol analysis is carried out by direct injection of the sample (fat) dissolved in hexane, without previous treatment and losses of this. Referring to phospholipids, only one study in this kind of sample exists in the literature, in which a method for the determination of these compounds is developed and a characterization of the different molecular species of phospholipids of subcutaneous fat from Iberian pig is done.[11] However, no study about the influence of diet on phospholipids from Iberian pig has been found in the literature.

The other method used for the authentication of the Iberian pig fattening diet has been near-infrared spectrometry (NIR). Although this technique is very simple, fast, and nondestructive and presents a low cost analysis, the different fattening systems are not perfectly differentiated (percentage of classification less than 100%).[12−14] Therefore, this is a disadvantage of this versus triacylglycerol analysis; beside of that, this last gives more information about the sample chemical composition. Because of the increase of the final fattening period with formulated feeds in an intensive regimen, the Spanish government has established new regulations to fix quality standards of Iberian pig products.[15] In food controls, efficient methods are required to prevent the wrongful use of the commercial name of higher quality products. In this realm, the triacylglycerols profile may provide an useful tool to authenticate Iberian pig products from different fattening systems.

In this work, triacylglycerols in a large number of Iberian pig subcutaneous fat samples from different campaigns have been determined by gas chromatography with flame ionization detection (GC-FID). Differences in the composition of the triacylglycerols profile have been used to differentiate between intensive and extensive fattening diet systems. Using them as chemical descriptors, pattern recognition (PR) techniques, such as principal component analysis (PCA), linear discriminant analysis (LDA), and soft independent modeling of class analogy (SIMCA), have been applied.

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MATERIALS AND METHODS

Chemicals and Reagents. Trilinolein (LLL) (537-40-6), triolein (OOO) (122-32-7), tripalmitin (PPP) (555-44-2), and tristearin (SSS) (555-43-1) were obtained from Sigma Aldrich Fluka (Steinheim, Germany). Standard solutions of 1.0% (m/v) of the triacylglycerols were prepared by dissolving them in analytical reagent grade *n*-hexane (Romil, Cambridge, United Kingdom).

Fat Samples. A total of 1995 samples of subcutaneous fat from castrated male pure Iberian pigs from the campaigns corresponding to the years 2003, 2004, 2005, 2009, and 2011 (Table 1) were analyzed.

Table 1. Analyzed Iberian Pig Subcutaneous Fat Samples

	campaigns				
fattening diet system	2003	2004	2005	2009	2011
extensive	592	595	367	176	182
intensive	4	5	0	32	42

Nineteen hundred thirteen samples were from animals fed with a fattening diet based exclusively on acorn (*Quercus ilex*, *Quercus suber*, and *Quercus faginea*) and pasture in an extensive system. Eighty-three samples corresponded to animals fed with concentrated feed in an intensive system. Samples were kindly provided by the Designations of origin "Jamón de Huelva", "Los Pedroches", and INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria). They were taken from different zones of production located in the southwest of Spain (Huelva, Cádiz, Sevilla, Cáceres, Badajoz, Salamanca, Córdoba, and Málaga). The animals were classified in the extensive and intensive groups according to the field notes taken by the veterinary inspector of the Designations of Origin and INIA during the final fattening period.

Apparatus and Methods. *Extraction of the Subcutaneous Fat.* Two samples were taken from each sacrificed animal according to a normalized procedure described in the literature.[16] Briefly, the procedure consisted of cutting a piece of approximately 3 cm × 3 cm at least 6 mm thick from the rump, at about 10 cm from the tail following the line of the back and containing skin, adipose tissue, and some loin. At the laboratory, the skin and the loin were carefully removed.[16] All of the chunks corresponding to each animal of a lot of sacrifice were punctured and homogenized before extraction. The representative sample of the lot was then obtained by melting the fat in a microwave oven, where it remained for 3 min at 360 W.[17] The samples of fat were filtered, and 0.05 mg of fat was dissolved in 1.5 mL of *n*-hexane for the GC analysis. Three replicates were determined for each sample.

Determination of the Triacylglycerols by GC-FID. Triacylglycerols were analyzed and identified as described previously by GC[3] in a Varian 3800 gas chromatograph (Varian Co., Palo Alto, CA) using a DB-17HT (Agilent J&W, Loveland, CO) fused silica capillary column (30 m long × 0.25 mm i.d., 0.15 μm film thickness). The oven temperature was kept at 320 °C and was then raised to 350 °C at a rate of 2.0 °C/min and held isothermally for 10 min. The injector temperature was kept at 360 °C, while the detector temperature was 370 °C. Hydrogen (2.1 mL min^{-1} column constant flow) was used as the carrier gas, and the makeup gas was nitrogen. Aliquots of 2 μL were injected.

The identification of 17 triacylglycerols species was carried out by means of standards of LLL, OOO, PPP, and SSS and by comparison with the relative retention times described previously in the literature.[3] Table 2 includes the different triacylglycerols species identified in the chromatograms shown in Figure 1. Palmitindioleine was used as a reference to calculate the relative retention times.

Data Analysis. The 17 triacylglycerols were used as chemical descriptors, and their peak areas were used as analytical signals. The quantification of each one was carried out by evaluating the corresponding relative percentage according to the normalization area procedure, assuming an equal factor response for any species. A data matrix whose rows are the samples and columns the variables was built. Each element of this matrix x_{ij} corresponds to the content of the triacylglycerol j for the sample i. The PR calculations were made by using the statistical package CSS:STATISTICA from Stafsoft (Tulsa, OK).

Table 2. Triacylglycerols Identified in Fat Samples[a]

peak	triacylglycerol	T_{RR}
1	PPP	0.65
2	MOP	0.67
3	PPS	0.79
4	POP	0.81
5	POPo + PLP	0.84
6	PLPo + MLO	0.87
7	PSS	0.95
8	PSO	0.97
9	POO	1.00
10	PLO	1.03
11	PLL + PoLO	1.07
12	SOS	1.14
13	SOO	1.17
14	OOO	1.19
15	SOL	1.21
16	OOL	1.24
17	OLL	1.29

[a]P, palmitic; Po, palmitolic; S, stearic; M, miristic; O, oleic; and L, linoleic.

RESULTS AND DISCUSSION

Fat Samples Analysis. Table 3 shows the mean and standard deviation values for the triacylglycerols determined in the analyzed fat samples corresponding to the five different campaigns considered and grouped according to the two fattening systems. The obtained results for the different species of triacylglycerols are in good agreement with those reported by other authors.[4] Median and ranges of all of the samples and groups of extensive and intensive fattening systems are included in Table 4. As can be seen, the most abundant triacylglycerol is POO, with median values of 32.02%. Other major triacylglycerols are PSO, OOO, and PLO, with median values of 13.92, 10.49, and 10.32%, respectively. POP, POPo + PLP, SOO, SOL, and OOL present medians that range between 3.7 and 6.62%. The remaining compounds range from 0.29% for PPP to 1.29% for SOS. In the case of PLO, SOO, OOO, SOL, OOL, and OLL, samples obtained from pigs fed an extensive system present higher median values than those from intensive fattening. The median contents in the remaining triacylglycerols are higher in intensive fattening samples. To find out significant differences between the two types of fattening systems, the Mann−Whitney U test was performed.[18] The statistical parameter U was obtained for each compound, and the respective z values were calculated to be compared with the z value in the normalized standard distribution for 95% confidence (z = 1.96). Results of application of this test are also shown in Table 4. It can be observed that the obtained z values are higher than the critical one for most of the considered variables. Only PLPo + MLO and PLL + PoLO do not present significant differences between intensive and extensive fattening diets. The highest differences are found for OOL and OLL, with z values up to 15. Other triacylglycerols present z values up to 10 (in absolute value), except POO, SOS, and the above-mentioned PLPo + MLO and PLL + PoLO.

In light of such results, it can be concluded that the two types of fattening diets show enough differences to make a more

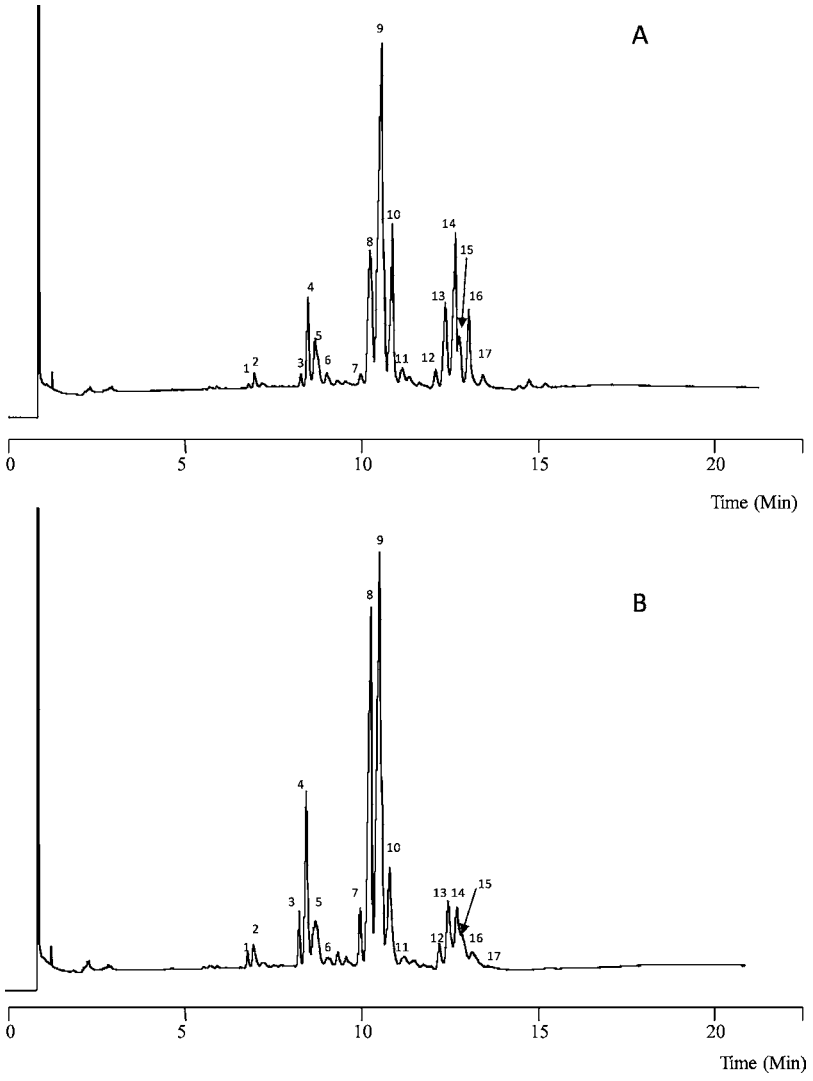

Figure 1. Chromatograms of the triacylglycerols profile of Iberian pig subcutaneous fat samples: (A) extensive fattening system and (B) intensive fattening system. See Table 1 for peak identification.

detailed study worthwhile. With the aim of finding out differences between the extensive and the intensive fattening systems, PR techniques were applied to the data. PLPo + MLO and PLL + PoLO were excluded according to the results of the Mann−Whitney U test.

Differentiation of Extensive and Intensive Fattening Systems. *PCA.* PCA is applied to the data set to obtain linear combinations of the variables called principal components (PCs). The first PC (PC1) expresses the largest variability, and each successive PC represents as much of the residual variability as possible. Three-dimensional plots of the PCs can be used to reveal the internal structure of the data and visualize the data trends.[19] In this case, the first three PCs obtained explain 54.3, 15.3, and 11.9% of the original variance, respectively. Figure 2 shows the distribution of the samples in the space defined by the three first PCs. It can be observed that the samples belonging to the different types fattening appear separated in this space, with PC1 being the most differentiating factor. The loadings of the variables in the three extracted PCs are shown in Table 5. As can be seen, PC1 is highly influenced by PPP, MOP, PPS, POP, PSS, PSO, OOO, and OOL. In the case of PC2, the most contributing variables are POO and SOO, while PC3 is correlated with PLO. Taking into account these results, LDA was applied to obtain an adequate classification model.

LDA. LDA computes linear combinations of the data to form discriminant functions (DFs), aiming for the separation of categories by the minimization of the within-class and between-class ratio of the sum of squares. The model can be constructed through a stepwise approach, which selects only the most discriminating variables. In this way, LDA can be used to reduce the number of chemical descriptors to be used in the characterization of classes.[20] In this case, the LDA model was built using the most contributing variables found by PCA. By applying a backward stepwise (BS) approach, a single DF was computed as a linear combination of MOP, PPS, PSO, OOL, and OLL. Figure 3 shows the distribution of the sample according to their scores for the DF. Samples from both feeding systems appeared overlapped in the range −3 to −1 of DF. Actually, the LDA model shows a recognition ability of 100% for intensive fattening samples and 94% in the case of extensive fattening.

SIMCA. As LDA is a hard modeling technique, each case is included in one of the considered classes. A soft modeling technique could give a more reliable classification model because it allows samples to pertain to one of the classes or not

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Table 3. Triacylglycerols Content (%)[a] of Iberian Pig Subcutaneous Fat Samples Grouped by Fattening Systems and Campaigns

		PPP	MOP	PPS	POP	POPo + PLP	PLPo + MLO	PSS	PSO	POO	PLO	PLL + PoLO	SOS	SOO	OOO	SOL	OOL	OLL
								2003										
EF (n = 592)	mean	0.25	0.85	0.80	5.68	4.76	0.87	1.10	15.04	31.16	10.00	1.43	1.42	6.83	9.96	3.71	5.17	0.96
	SD	0.06	0.15	0.20	0.88	0.46	0.10	0.29	2.07	1.17	0.64	0.20	0.18	0.44	2.09	0.27	1.05	0.23
IF (n = 4)	mean	0.42	1.29	1.30	8.32	5.97	0.82	1.83	20.07	30.76	9.82	1.18	1.53	5.91	5.08	3.00	2.37	0.33
	SD	0.06	0.17	0.15	0.64	0.40	0.07	0.12	1.06	0.66	0.54	0.06	0.16	0.49	0.55	0.22	0.41	0.05
								2004										
EF (n = 595)	mean	0.19	0.76	0.68	5.16	4.64	0.79	0.99	14.26	31.09	10.83	1.46	1.30	6.69	10.47	3.79	5.89	1.04
	SD	0.06	0.14	0.17	0.74	0.55	0.16	0.25	1.70	1.8	0.71	0.33	0.17	0.43	1.79	0.33	0.92	0.25
IF (n = 5)	mean	0.35	1.25	1.16	7.89	5.49	0.84	1.49	18.75	32.07	9.74	1.27	1.41	6.09	5.81	3.24	2.80	0.34
	SD	0.05	0.22	0.09	0.88	1.99	0.05	0.13	0.61	2.37	0.64	0.29	0.09	0.25	0.29	0.09	0.14	0.05
								2005										
EF (n = 367)	mean	0.15	0.61	0.55	4.71	2.51	0.69	0.78	12.42	36.05	9.84	1.36	1.10	6.61	11.38	3.97	6.10	1.17
	SD	0.10	0.16	0.17	0.84	0.80	0.19	0.25	1.80	2.75	1.21	0.29	0.23	0.53	1.94	0.63	1.06	0.32
IF (n = 0)	mean																	
	SD																	
								2009										
EF (n = 176)	mean	0.25	0.83	0.79	5.53	2.78	0.77	1.07	13.97	34.95	11.31	2.00	1.15	5.81	9.27	2.98	5.25	1.29
	SD	0.14	0.32	0.34	1.48	0.50	0.18	0.36	2.33	1.31	1.24	0.61	0.21	0.84	2.94	0.34	1.24	0.30
IF (n = 32)	mean	0.45	1.25	1.54	8.40	2.92	0.70	2.01	21.18	35.44	9.27	1.49	1.48	5.24	4.22	2.27	1.90	0.21
	SD	0.11	0.23	0.27	0.95	0.45	0.12	0.34	1.81	0.88	0.90	0.30	0.27	0.71	0.81	0.24	0.36	0.05
								2011										
EF (n = 182)	mean	0.18	0.68	0.68	4.98	4.45	0.84	0.95	13.56	35.04	10.44	1.89	1.24	5.93	9.45	3.59	5.05	1.04
	SD	0.06	0.15	0.21	0.77	0.53	0.16	0.37	2.47	1.27	1.19	0.42	0.24	0.61	1.72	0.43	1.02	0.42
IF (n = 42)	mean	0.38	1.16	1.29	7.70	5.45	0.84	1.60	18.82	35.49	8.60	1.42	1.55	5.59	4.99	2.79	2.08	0.26
	SD	0.09	0.20	0.29	0.93	0.66	0.13	0.41	2.09	1.43	0.74	0.15	0.29	0.77	1.09	0.39	0.38	0.07

[a]Obtained from triplicate analysis.

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Table 4. Median and Ranges of Triacylglycerols (%) and Results of the Mann−Whitney U Test[a]

triglyceride	all samples	extensive fattening	intensive fattening	U	z
PPP	0.19 (0.03−0.77)	0.19 (0.03−0.77)	0.39 (0.22−0.66)	8452	13.80
MOP	0.75 (0.16−1.71)	0.74 (0.16−1.59)	1.18 (0.72−1.71)	9958	13.51
PPS	0.68 (0.03−2.08)	0.67 (0.03−1.59)	1.34 (0.67−2.08)	5790	14.32
POP	5.15 (0.39−10.07)	5.11 (0.39−8.74)	8.06 (6.25−10.07)	4444	14.58
POPo + PLP	4.43 (1.06−6.86)	4.42 (1.06−6.86)	4.91 (1.83−6.76)	60910	3.59
PLPo + MLO	0.79 (0.16−1.59)	0.79 (0.16−1.59)	0.78 (0.37−1.13)	75840	−0.68
PSS	0.96 (0.19−2.67)	0.95 (0.19−2.42)	1.76 (0.74−2.67)	11618	13.18
PSO	13.92 (6.98−24.72)	13.81 (6.98−22.49)	20.18 (13.68−24.72)	6686	14.14
POO	32.02 (0.77−42.85)	31.93 (0.77−42.85)	35.26 (30−40.21)	38740	7.90
PLO	10.32 (6.87−17.94)	10.36 (6.87−17.94)	9 (6.99−11.51)	23351	−10.90
PLL + PoLO	1.43 (0.54−3.26)	1.43 (0.54−3.26)	1.44 (1−2.17)	73600	−1.12
SOS	1.29 (0.49−2.39)	1.28 (0.49−2.17)	1.46 (0.91−2.39)	38835	7.89
SOO	6.62 (4.13−9.38)	6.64 (4.13−9.38)	5.45 (4.25−7.32)	22344	−11.10
OOO	10.49 (3.15−17.02)	10.62 (4−17.02)	4.66 (3.15−7.88)	2602	−14.94
SOL	3.7 (0.6−7.17)	3.72 (0.6−7.17)	2.55 (1.81−3.55)	7153	−14.05
OOL	5.63 (1.26−9.7)	5.66 (2.37−9.7)	2.04 (1.26−2.95)	192	−15.41
OLL	1.03 (0.09−2.18)	1.04 (0.36−2.18)	0.25 (0.09−0.4)	13	−15.44

[a]Nonsignificant differences for $z < 1.96$.

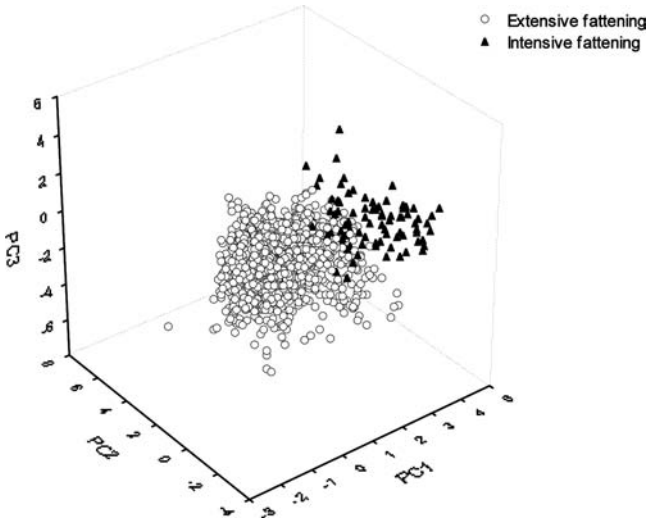

Figure 2. Score plot in the space of the three first PCs.

Table 5. Loadings of the Variables in the Three First PCs

variable	PC1	PC2	PC3
PPP	0.851	−0.105	−0.134
MOP	0.876	−0.089	−0.267
PPS	0.961	−0.05	−0.038
POP	0.952	−0.134	−0.117
POPo + PLP	0.417	0.452	−0.516
PSS	0.882	0.131	0.063
PSO	0.929	0.177	0.108
POO	−0.204	−0.744	0.573
PLO	−0.264	−0.293	−0.806
SOS	0.618	0.656	0.214
SOO	−0.326	0.834	0.35
OOO	−0.879	0.247	0.137
SOL	−0.471	0.358	−0.316
OOL	−0.914	0.182	−0.203
OLL	−0.765	−0.082	−0.296

to be included in any of them. In this way, SIMCA is one of the most useful PR techniques that determines the number of PCs needed to describe the structure of each class. The boundaries of each class are obtained, and objects are included in the class if they fall into the *n*-dimensional box limited by these boundaries.[20] In this case, SIMCA was applied to differentiate extensive and intensive fattening systems by using the same variables retained by LDA. The results of this model are shown in the Coomans' plot (Figure 4). As can be seen, most of the extensive fattening samples appear into the limits of their class. Intensive fattening samples are located in the overlapping area of both classes, at the bottom left corner of the plot. They have high probabilities to be included in both classes, but most of them present the highest probability to pertain to the intensive fattening class. A number of samples appear out of the limits of the classes, and this means that they are not assigned to any of two considered fattening systems. For the class extensive fattening, a recognition ability of 89.8% was accomplished, while 0.4% of the samples were wrongly classified as intensive fattening and 9.8% were not included in any of the classes. For the samples of intensive fattening system, 85.6% were correctly assigned, 10.8% were included in the extensive fattening class, and 3.6% were not assigned to any of the classes.

Variable−Variable Plot. Variable−variable plots are the simplest way to classify samples. Once the most discriminating variables have been found, the number of possible combinations of two variables can be reduced. Considering the variables extracted by BS-LDA, that is, MOP, PPS, PSO, OOL, and OLL, 10 different variable−variable plots can be depicted. Figure 5 shows the sample distribution in the plane defined by OLL and OOL. Boundaries appearing in this figure have been obtained as the average value of OLL and OOL plus 1.96 ($P = 0.05$) times their standard deviation. It can be seen that samples from intensive fattening present OOL and OLL contents lower than 2.90 and 0.38%, respectively. Samples with both values under these limits could be classified as an intensive fattening system.

In conclusion, the contents in 17 triacylglycerols have been determined in samples of subcutaneous fat of Iberian pig fed two different fattening systems, intensive and extensive. A study on the discriminating power of these compounds has been performed to classify samples according to the pig fattening system. Initially, these variables were tested to find out statistical differences between the two classes by means of

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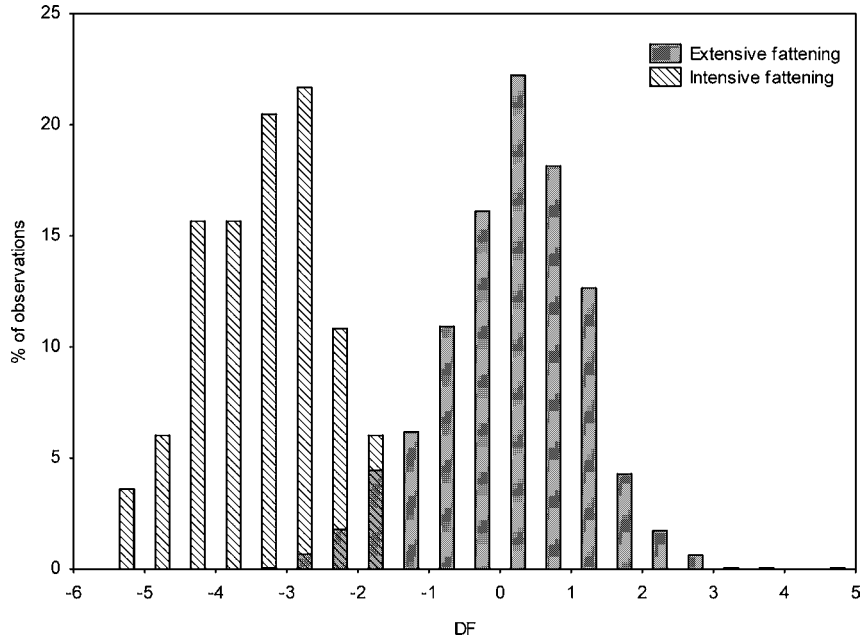

Figure 3. Distribution of the samples according to their scores for the discriminant function.

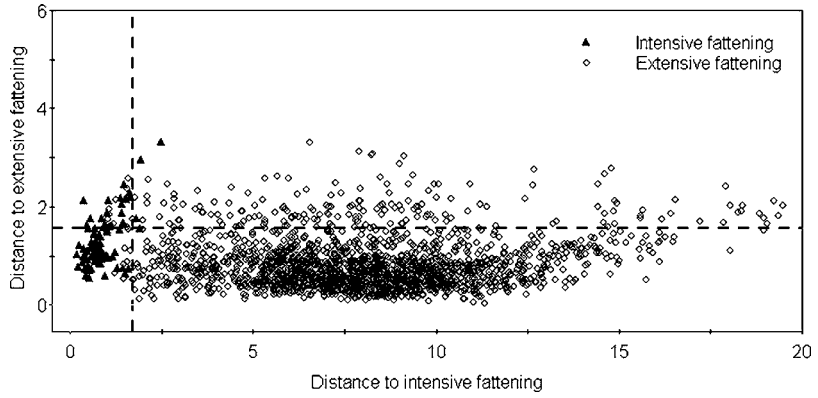

Figure 4. Coomans' plot.

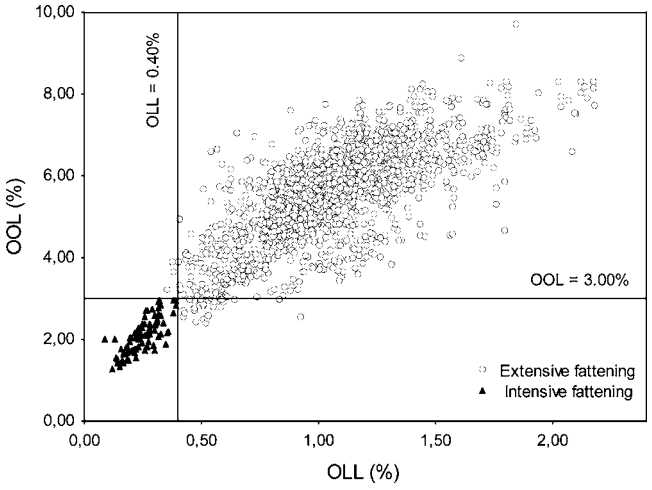

Figure 5. Variable−variable plot OOL−OLL.

the Mann−Whitney U test. Samples from extensive and intensive fattening presented significant differences in 15 of the determined triacylglycerols. After PCA was applied, natural groupings of samples from the same type were observed in the space of the three first PCs. The most contributing variables to these PCs were then selected to perform a BS LDA obtaining a classification model with 94.24% of overall recognition ability. The model retained the most discriminating variables, such as MOP, PPS, PSO, OOL, and OLL. By using a variable−variable plot of OOL and OLL, a very easy and useful way to visualize the trends of the samples can be obtained. Samples from intensive fattening system present values below 0.40% of OLL and 3.00% of OOL, and unknown samples could be classified as belonging to this class only if both conditions are met.

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Notes

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