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Characterization and quantification of 4-methylsterols and 4,4-dimethylsterols from Iberian pig subcutaneous fat by gas chromatography–mass spectrometry and gas chromatography–flame ionization detector and their use to authenticate the fattening systems

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ABSTRACT

4-Methylsterols and 4,4-dimethylsterols of 47 samples of subcutaneous fat from Iberian pigs reared on two different fattening systems, "*Extensive*" and "*Intensive*", have been analyzed by GC–MS and GC–FID. The lipids were extracted by melting the subcutaneous fat in a microwave oven. The unsaponifiable matter was fractionated by thin layer chromatography. Then, the analysis was performed on a capillary SPB-5 column (30 m × 0.25 mm i.d., 0.15 μm film thickness), with hydrogen as a carrier gas and using a flame ionization detector. n-eicosanol was used as internal standard for quantification of individual methylsterols. These compounds have been analyzed by GC–MS for their identification. The full scan of free and trimethyl silyl ethers was used as acquisition mode. Six compounds have been identified for the first time in this type of samples: (3β,4α,5α)-4-methyl-cholesta-7-en-3-ol, (3β,4α,5α)-4-methyl-cholesta-8(14)-en-3-ol, (3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol, (3β)-lanosta-8,24-dien-3-ol, (3β, 5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol and (3β)-lanost-9(11),24-dien-3-ol. The samples were derivatized as trimethyl silyl ethers before their analysis by GC–FID.

By using these compounds as chemical descriptors, pattern recognition techniques were applied to differentiate between extensive and intensive fattening systems of Iberian pig. Several pattern recognition techniques, such as principal component analysis, linear discriminant analysis, support vector machines, artificial neural networks, soft independent modeling of class analogy and k nearest neighbors, have been used in order to find out a suitable classification model. A multilayer perceptron artificial neural network based on the contents of the above mentioned compounds allowed the differentiation of the two fattening systems with an overall classification performance of 91.7%.

1. Introduction

The Iberian pig is an autochthonous breed grown in Iberian Peninsula, whose productive system is closely linked to the Mediterranean silvopastoral environment in which it lives for centuries (called *La Dehesa*). The outdoor rearing system is associated with an increase in animal welfare, reduced environmental impact and protection of a traditional production system.

An interesting characteristic of this race is associated to its great capacity to accumulate fat under its skin and between the muscular fibers. This fat is what makes its products so appreciated by consumers, since it is responsible for its aroma, taste and texture. The quality of these depends on the final fattening diet of the animal. So, several authors have studied the chemical composition of subcutaneous fat in the function of this final fattening type, period and system. The profiles of fatty acids [1,2], triacylglycerols [3–5], hydrocarbons [6–8] and volatile compounds [9,10] have been related with the fattening diets of Iberian pigs and can be used as chemical descriptor to differentiate between the different feeding backgrounds of animals. Near-infrared spectrometry (NIR) is another method that has been used for the authentication of this animal fattening diet [11–13]. This is a very simple, fast, cheap and nondestructive technique, but it does not differentiate perfectly between the different fattening systems and does not give information about the sample chemical composition.

However, other compounds of subcutaneous fat, as it is in the case of 4-methylsterols and 4,4-dimethylsterols, have not been used in this sense.

4,4-Dimethylsterols have steroid structure and are present in all vegetable fats. The most characteristic is the cycloartenol

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(9β,19-cyclo-5α-lanost-24-en-3β-ol). Cycloartanol (9β,19-cyclo-5α-lanostan-3β-ol), 24-methylcycloartanol (24-methylen-9β,19-cyclo-5α-lanost-24-ene-3β-ol), α-amyrin, β-amyrin and butyrospermol are also present in these fats [14,15].

Methylsterols can be used in characterization of vegetable oils [16], these compounds being usually determined by gas chromatography or high performance liquid chromatography [17].

The determination of these compounds, as it has been reported in the literature, consists of a separation of this fraction by thin layer chromatography and its subsequent analysis by gas chromatography [16,17]. Analysis of sterols is possible by high performance liquid chromatography (HPLC), using UV and mass spectometric (MS) detection for their quantification and identification, respectively. Some authors have developed a HPLC/atmospheric pressure chemical ionization–MS method for the analysis of these compounds, in which no previous derivatization is needed [18,19]. Riddle and Guiochon [20] studied several stationary reverse phases (C8, C18, zirconia-based adsorbent), obtaining that the graphitic carbon phase produced the best separation of sterols and that increasing the column temperature accelerates their elution markedly. Careri et al. [18] also found a better separation of these compounds using a C8 column. However, different mobile phases have been used for this purpose.

The aim of the present study has been, in the first place, to focus on the complete identification of the 4-methylsterol and 4,4-dimethylsterol fractions of the subcutaneous fat from Iberian pig. Secondly, the contents of these compounds have been used as chemical descriptors to differentiate extensive and intensive fattening systems. With this aim, several pattern recognition (PR) techniques, such as principal component analysis (PCA), linear discriminant analysis (LDA), support vector machines (SVM), artificial neural networks (ANN), soft independent modeling of class analogy (SIMCA) and k nearest neighbors (KNN), have been applied.

2. Experimental

2.1. Reagents and standards

Diethyl ether and ethanol 96 vol%, both for analysis grade, were supplied by Prolabo (Paris, France). Potassium hydroxide 85% pellets and anhydrous sodium sulfate, both for analysis grade, were obtained from Panreac (Barcelona, Spain). Chloroform for HPLC grade and n-Hexane Super Purity Solvent grade were provided by ROMIL (Cambrige, UK). 2,7-Diclorofluorescein for analysis grade, was supplied by Fluka Chemical Co. (Ronkonkoma, NY, USA). n-eicosanol (Sigma Chemical Co. St. Louis, Mo, USA) was used as an internal standard. A mixture 9:3:1 (v/v/v) of anhydrous pyridine (Fluka), hexamethyldisilazane (Fluka) and trimethylchlorosilane (Fluka) was used as a derivatazing reagent. TLC Silica gel 60, plates $20 \times 20\ cm^2$ were supplied by Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Instrumentation

4-Methylsterol and 4,4-dimethylsterol fractions were analyzed in an Agilent (Palo Alto, CA, USA) 7890A gas chromatograph equipped with a cold-on column injector and a flame ionization detector; a capillary SPB-5 column (30 m × 0,25 mm i.d., 0,25 μm film thickness, Supelco, Bellefonte, PA, USA) and an Agilent G 4513A automatic injector were used. The oven temperature was kept at 90 °C and was then raised to 220 °C at a rate of 45.0 °C min^{-1} and held isothermally for 1.0 min. It was then raised to 270 °C at a rate of 2.5 °C min^{-1}. Finally, it was raised to 290 °C at a rate of 3.0 °C min^{-1} and held isothermally for 4.44 min. The operating condition of injector was "Oven Track" mode, where the injector temperature tracks the column oven temperature automatically at 3 °C higher than the oven temperature. The detector temperature was 320 °C. Hydrogen was used as the carrier gas at 1.6 mL min^{-1} in constant flow mode. Air and hydrogen with flow rates of 450 and 40 mL min^{-1}, respectively, were used for the detector, which had an auxiliary flow of 40 mL min^{-1} of nitrogen.

In order to identify the 4-methylsterols and 4,4-dimethylsterols fractions a GC–ion-trap-MS experiment was performed using a Varian-CP3800 gas chromatograph coupled to a Saturn 2000 ion trap mass spectrometer (Varian, Palo Alto, CA, USA) equipped with a CP8400 autosampler operating in full scan mode from 40 to 550 amu at 1 scan/s. The column used was a DB-5 MS (J&W Scientific, Albany, NY, USA) fused silica capillary column (30 m long × 0.25 mm i.d × 0.25 μm film thickness). The GC conditions included hydrogen as the carrier gas at 1.8 mL min^{-1} in constant flow mode. The oven temperature was kept at 220 °C for 3.0 min, then was raised to 290 °C at a rate of 3.0 °C min^{-1} and held isothermally for 3.67 min. The injector temperature was 310 °C. Split injection mode was used with a ratio of 1:8 and the injection volume was 1 μL.

The MS operating conditions were the following: Ion source and transfer line temperatures were 190 and 290 °C, respectively. The electron energy was 70 eV with a resolution of 1 and the emission current 10 μA; dwell time and inter-channel delay were 0.08 s and 0.02 s respectively. Varian Mass Spectrometry Workstation version 6.30 software was used for data acquisition and processing of the results.

2.3. Samples and sample treatment

A total of 47 samples of subcutaneous fat from castrated male 14 month old pure Iberian pigs were analyzed. Twenty seven of them correspond to animals fed in an extensive fattening (EF) diet and 20 in intensive fattening (IF) system. Samples were provided by the different Designations of Origin located in the southwest of Spain. The samples were obtained following the method proposed by the Spanish regulation [21]. Table S1 of the supplementary electronic material shows the identification code assigned to each one.

4-Methylsterol and 4,4-dimethylsterol fractions were extracted as it has been described before in literature [22]. 5.0 + 0.1 g of fat was weighted and 0.5 mL of 1-eicosanol solution in chloroform (0.01% m/v) was added. Then, the sample was saponified for 30 min with 50 mL of 2 M ethanolic potassium hydroxide. The solution was passed into a 500 mL decanting funnel, 100 mL distilled water was added and the mixture was extracted twice with three 80 mL portions of diethyl ether. The organic extracts were combined in another funnel and were washed several times with 100 mL portions of water, until the wash reached neutral pH. The ether solution was dried over anhydrous sodium sulfate and then evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure.

The complete unsaponifiable fraction was dissolved in approximately 1 mL of chloroform and the solution was spotted on a TLC plate previously impregnated with 0.2 M ethanolic potassium hydroxide and dried for 1 h to 100 °C. The plate was developed two times using hexane–diethyl ether (65:35, v/v) and subsequently dried. After this, it was sprayed with the 2,7-dichlorofluorescein solution and the pink bands of the methylsterols can be observed under UV light. This band was scraped-off and methylsterols were dissolved into 10 mL of chloroform and 10 mL of diethyl ether. The solution obtained was filtered-off through a paper filter. The solvent was evaporated to dryness under reduced pressure.

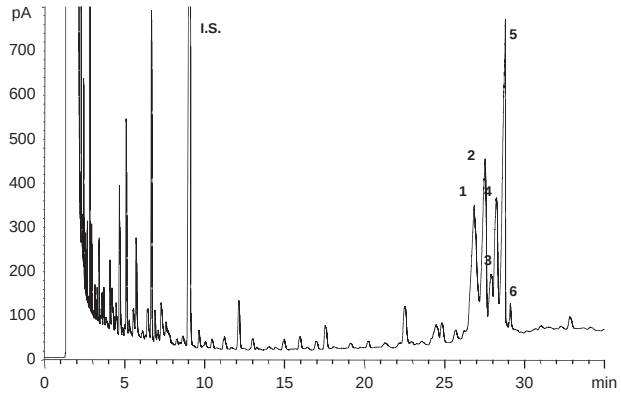

Fig. 1. Chromatograms of 4-methylsterols and 4,4-dimethylsterols obtained from the subcutaneous fat of Iberian pig.

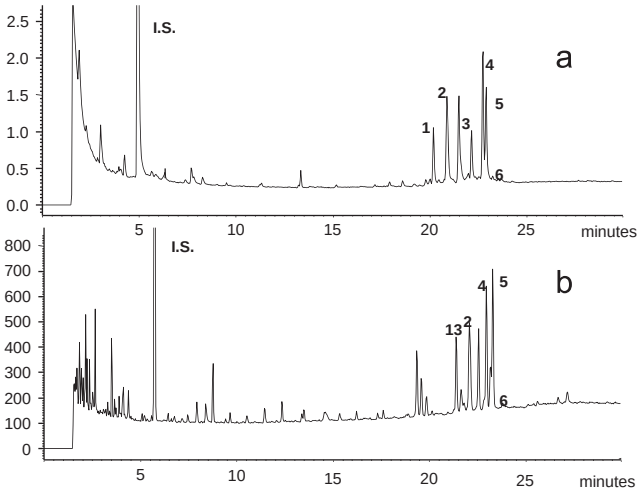

Fig. 2. GC–ion trap-MS chromatogram in full scan mode of 4-methylsterols and 4,4-dimethylsterols isolated. For peak assignments see Table 2. A: free compounds; and B: trimethyl silyl derived.

The methylsterolic fraction was treated with 0.15 mL of the derivatizing reagent to obtain the trimethyl silyl (TMS) derivates. Previously the free methylsterolic fraction was analyzed by GC–MS.

2.4. Quantitative analysis and statistical treatment

The peak area of the analyte was used as an analytical signal. The quantification of individual methylsterols was carried out using l-eicosanol as internal standard. The response factor relative to 4-methylsterols and 4,4-dimethylsterols is close to the unit. A representative chromatogram report of subcutaneous fat and the corresponding peak identification are shown in Fig. 1.

The methylsterols identified were considered as chemical descriptors. A datamatrix, whose rows are the samples and whose columns are the variables, was built. Each element of this matrix x_{ij} corresponds to the content of methylsterol j for the sample i. Mann–Whitney U-test has been performed in order to highlight significant differences in the methyl-sterols contents of both fattening systems. PCA was used to visualize the distribution of the data as well as to detect most influential variables to natural group separations. LDA was used to reduce the number of variables to be used to build classification models. Several modeling techniques have been applied, such as LDA, SVM, MLP–ANN, SIMCA and KNN. The operation of these techniques as well as their suitability for solving the classification problem has been treated in the section of results and discussion. PR calculations were made by using the statistical package STATISTICA 8.0 from Statsoft (Tulsa, OK, USA). SIMCA calculations were carried out by using SIMCA-P 9.0 from Umetrics AB (Umeå, Sweden).

3. Results and discussion

3.1. Identification of components

Six compounds have been identified in the 4-methylsterol and 4,4-dimethylsterol fractions of the subcutaneous fat from Iberian pig. Fig. 2 shows the GC–ion trap-MS chromatogram in full scan mode of the 4-methylsterol and 4,4-dimethylsterol fractions isolated by the TLC. Identification of peaks has been carried out by GC–ion-trap-MS comparing the spectra with those from NIST (National Institute of Standards and Technology) and Wiley libraries. Fig. 2A corresponds to GC–ion trap-MS of the free 4-methylsterol and 4,4-dimethylsterol fractions. Fig. 2B corresponds to GC–ion trap-MS of their trimethyl silyl ethers. Table S2 of the supplementary electronic material shows the retention times, the base peak, the molecular ion and the more characteristic ions for free compounds and their trimethyl silyl ethers.

The peaks with retention time of 20.19 in Fig. 2A and 21.24 in Fig. 2B show the mass spectra that appear in Fig. S1A and B respectively and of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β,4α,5α)−4-methyl-cholesta-7-en-3-ol. This compound is *Lofenol*, which is an intermediate in the biosynthesis of cholesterol (and other sterols) in plants from cycloartenol [23]. Pepper seed oil is characterized by a high content in lofenol, approximately 30% of the 4-methylsterol fraction [24]. It has also been described in the *Symphorzcarpus albus* leaves [25]. It is reported for the first time in the subcutaneous fat of Iberian pigs.

The peaks with retention time of 20.89 in Fig. 2A and 21.93 in Fig. 2B show the mass spectra that appear in Fig. S2A and B respectively of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β,4α,5α)−4-methyl-cholesta-8(14)-en-3-ol. This compound is *4α-methyl-zimostenol*; it is a 4-methylsterol located in some seed oils, such as the *Capsicum annuum* seed [26]. It is reported for the first time in the subcutaneous fat of Iberian pigs.

The peaks with retention time of 21.48 in Fig. 2A and 22.40 in Fig. 2B show the mass spectra that appear in Fig. S3A and B respectively of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol. This compound is an intermediate in the biogenesis of sterols, concretely; it has been described in the biosynthesis of ergosterol in fungi and cholesterol in animals from lanosterol [27]. Its stimulant activity of meiosis and its accumulation in ovaries and testicles have been also described [28]. It is reported for the first time in the subcutaneous fat of Iberian pigs.

The peaks with retention time of 22.74 in Fig. 2A and 22.81 in Fig. 2B show the mass spectra that appear in Fig. S4A and B respectively of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β)-lanosta-8,24-dien-3-ol (lanosterol). This compound is the precursor of others steroids in animals and some protists [29]. It is one of the steroids predominant in muscle and adipose tissue of bovine [30]. It has been also described in skin and liver of rats [31–33]. This compound has been previously identified in the unsaponifiable fraction from pork fat [34] and in some plants, such as *S. albus* [25].

The peaks with retention time of 22.91 in Fig. 2A and 23.14 in Fig. 2B show the mass spectra that appear in Fig. S5A and B respectively of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β,5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol. This compound has been isolated and characterized as an intermediate in the biosynthesis of cholesterol [35]. It is reported for the first time in the subcutaneous fat of Iberian pigs.

The peaks with retention time of 23.53 in Fig. 2A and 23.64 in Fig. 2B show the mass spectra that appear in Fig. S6A and B respectively of the supplementary electronic material. According to these spectra, it is suggested that both peaks correspond to (3β)-lanosta-9(11),24-dien-3-ol (parkeol). This compound is a very interesting triterpenic alcohol since it is an intermediate in gramisterol and other sterols synthesis. It has been described in Spanish olive oils [16]. It is reported for the first time in the subcutaneous fat of Iberian pigs.

3.2. Differentiation of extensive and intensive fattening systems

Table 1 shows median and ranges of methylsterols corresponding to all samples and also those corresponding to EF and IF. As it can be observed, the most abundant compound was (3β,5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol, with the highest content in IF samples with a median value of 1.76 mg kg^{-1}. On the other hand, the less abundant methylsterol was (3β)-lanosta-9(11),24-dien-3-ol with 0.174 mg kg^{-1} median value. All compounds, except this last, showed the highest values in IF samples. In order to find out significant differences between the two types of fattening systems, the Mann–Whitney *U*-test was performed [36]. The statistical parameter *U* was obtained and a *z*-value was calculated and compared with the *z*-values in the normalized standard distribution for 95% confidence ($z=1.96$). The obtained results are also shown in Table 1. As can be seen the obtained *z*-values for all compounds were higher than 1.96 and then it can be concluded that there are significant differences between the methylsterols content in IF and EF samples. Considering these results a PR study was carried out.

PCA was applied to visualize data trends [37]. The three first principal components (PCs) were computed, explaining 52.45%, 19.37% and 13.25% of the total variance. The contribution of each variable to the PCs is shown in Table 2. As can be observed PC1 was highly influenced by the variables (3β,4α,5α)−4-methyl-cholesta-8(14)-en-3-ol, (3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol, (3β)-lanosta-8,24-dien-3-ol and (3β,5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol. In the case of PC2, the most contributing variable is (3β)-lanosta-9(11),24-dien-3-ol. PC3 does not present correlation up to 0.7 with any variable. Fig. 3 shows the distribution of the samples in the plane defined by the two first PCs (Fig. 3a) and in the plane formed by PC2 and PC3 (Fig. 3b). It can be observed that EF samples appear at positive values of PC1, whereas the IF samples appear at negative values. There are no remarkable differences according to scores of PC2 and PC3. Taking into account the distribution of samples, linear models could be used to differentiate the considered fattening systems and with this aim LDA was applied.

LDA computes linear combinations of the data to obtain discriminant functions (DFs) allowing separation of the categories by the minimization of the within-class and between-class ratio of the sum of squares. The model can be constructed through a

Table 2
Loadings of the variables in the three first PCs.

	PC1	PC2	PC3
(3β,4α,5α)-4-methyl-cholesta-7-en-3-ol	−0.573	−0.549	0.303
(3β,4α,5α)-4-methyl-cholesta-8(14)-en-3-ol	−0.890	0.216	0.090
(3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol	−0.785	−0.059	0.501
(3β)-lanosta-8,24-dien-3-ol	−0.768	0.124	−0.537
(3β,5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol	−0.904	0.114	−0.249
(3β)-lanosta-9(11),24-dien-3-ol	0.033	0.884	0.307

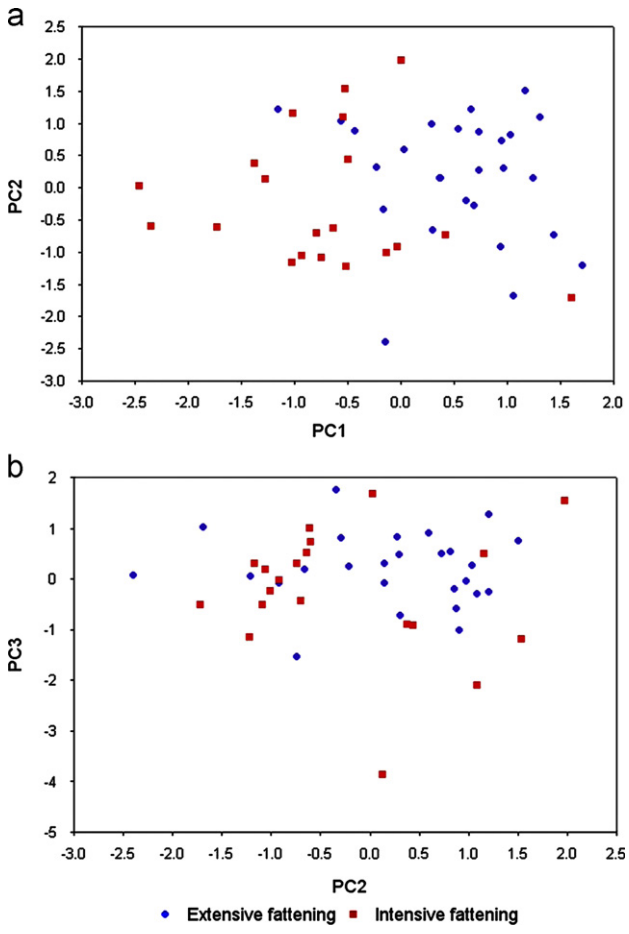

Fig. 3. Score plot in the plane of (a) PC1–PC2 and (b) PC2–PC3.

Table 1
Median and ranges of methylsterols (mg kg^{-1}) in samples from extensive (EF) and intensive (IF) fattening systems.

Compound	All samples	EF	IF	*U*	*Z*
(3β,4α,5α)-4-methyl-cholesta-7-en-3-ol	1.073 (0.412–2.517)	0.91 (0.41–2.52)	1.34 (0.78–2.16)	122.0000	−3.18445
(3β,4α,5α)-4-methyl-cholesta-8(14)-en-3-ol	1.054 (0.343–1.854)	0.89 (0.34–1.72)	1.16 (0.46–1.85)	130.0000	−3.01232
(3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol	0.293 (0.099–0.826)	0.27 (0.1–0.59)	0.39 (0.15–0.83)	126.0000	−3.09839
(3β)-lanosta-8,24-dien-3-ol	0.622 (0.344–1.829)	0.52 (0.34–0.91)	0.95 (0.39–1.83)	52.0000	−4.69061
(3β,5α)-4,4-dimethyl-cholesta-8,14-dien-3-ol	1.469 (0.702–2.156)	1.21 (0.7–1.95)	1.76 (0.75–2.16)	103.0000	−3.59327
(3β)-lanosta-9(11),24-dien-3-ol	0.174 (0.048–0.387)	0.2 (0.07–0.34)	0.15 (0.05–0.39)	171.0000	2.13014

Results of the Mann–Whitney *U*-test.

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 [38]. In this case, the LDA model was built applying a forward stepwise approach, a single DF was computed as linear combination of (3β,4α,5α)-4-methyl-cholesta-7-en-3-ol, (3β,4α,5α)-4-methyl-cholesta-8(14)-en-3-ol, (3β,5α)-4,4-dimethyl-cholesta-8(14),24-dien-3-ol, (3β)-lanosta-8,24-dien-3-ol and (3β)-lanosta-9(11),24-dien-3-ol. The datamatrix was divided into two subsets, a training set consisting of 75% of the cases and a test set formed with the remaining cases. The training set was used to establish the relationships between inputs and outputs and the model was evaluated by means of the results obtained for the test set. The samples were selected randomly to ensure that all the samples have the same probability of belonging to the training set and, consequently, that the constructed model will take into account the within class variability, learning the gross structure of the data. To evaluate the performance of a chemometric technique it is advisable to perform a cross-validation procedure to obtain different models with the same datamatrix, by using different subsets as training and test samples. These kinds of procedures allow the computation of a mean efficiency of the model for each considered class. In this work stratified delete-a-group jackknife (DAGJK) procedure [39] was applied to obtain nine replicates for the training and test sets, i.e., nine different LDA models were obtained to compute the mean classification efficiency for each class. See Table S3 of the supplementary electronic material to know the data division used to obtain these nine models. Stratified DAGJK works by randomly deleting a group of cases from each class, building the LDA model and using the deleted samples as a test set to compute its classification ability. The results are shown in Table 3. As can be seen 79.4% and 84.4% of classification performance were obtained for extensive fattening and intensive fattening respectively. In view of these results and to improve the efficiency of the classification model, non-linear approaches, based on support vector machines (SVMs) and artificial neural networks (ANNs), were considered.

SVMs are supervised learning techniques that separate the cases by an optimal hyperplane that maximizes the distances between classes. The boundaries are defined by the closest cases (support vectors) from the margins of the class. When no linear separation is possible in the input space, the original objects have to be rearranged in a new higher dimensional (feature) space, using a set of mathematical functions, in which a linear solution is possible. Once the boundaries are established, the back-projection of the optimal separating hyperplane to the original input space will result in a non-linear boundary. The optimal complexity of these boundaries is controlled by a parameter (penalty error) avoiding overtraining [40]. In this case, a SVM model was built by using a sigmoid function to obtain the feature space and the same previously described cross-validation procedure was applied. As can be seen in Table 3, 84.1% and 80.0% of extensive and intensive fattening samples were correctly classified, respectively. This results lead to approximately the same overall results (82.1%) as those obtained by the LDA model.

Table 3
Classification ability obtained with different models.

	EF	IF	Overall
LDA	79.4 ± 7.5	84.4 ± 16.7	81.9 ± 12.1
SVM	84.1 ± 4.8	80.0 ± 17.3	82.1 ± 11.0
MLP 6:7:2	85.7 ± 10.1	97.8 ± 6.7	91.7 ± 8.4
SIMCA	55.6 ± 16.7	80.0 ± 20.0	67.8 ± 18.3
KNN	81.7 ± 12	73.2 ± 14.6	77.5 ± 13.3

ANNs are sophisticated modeling techniques that simulate a biological nervous system and perform discriminant models and regression. They are especially useful when other statistical techniques are not able to predict complicated phenomena [41]. Here, multilayer perceptrons (MLP) ANNs were applied in order to obtain a more efficient classification model. MLP–ANNs are feedforward multilayer networks consisting of neurons arranged in layers (an input layer, various hidden layers and an output layer), being the connections (weights) unidirectional from input to output. This type of ANNs is usually trained by back propagation (BP) and needs a third set of samples, called verification set, to avoid overtraining. The random stratified DAGJK procedure was used to divide the datamatrix into training, verification and test sets. A BP–MLP–ANN model with 6:7:2 architecture was constructed and its results are also shown in Table 3. The model showed a classification ability of 85.7% for EF and 97.8% for IF system. As BP–MLP–ANN model improves the results obtained by LDA, non-linear models must be considered to differentiate the EF and IF systems according to the methylsterols content.

The obtained model has been also compared with other commonly used chemometric techniques, such as SIMCA and KNN. In both cases the same DAGJK procedure was used to obtain the method performance. SIMCA applies PCA for each class by using samples from the training set. The boundaries of the class are obtained and objects are classified in a group if they fall into the space limited by these boundaries [42]. The results obtained when SIMCA is applied to the discrimination of fattening systems according to the methylsterols profile of subcutaneous fat are shown in Table 3. As it can be seen, the classification ability for IF system (80%) is comparable to those results obtained by LDA and SVM, but the efficiency for EF is lower than those obtained for these techniques (55.6%). As SIMCA can be considered as a linear classification technique, the obtained results are expected according to the LDA results. As SIMCA is a soft modeling technique, some samples can be classified as not belonging to any of the classes. In this case, the model designed 9.5% and 6.7% of extensive and intensive fattening samples as "no class", respectively.

KNN is a nonparametric method used to classify unknown objects according to the class membership of the cases from the training set. The distance of each new case is computed to all the training cases and it is assigned to the class of the majority of its k nearest neighbors. KNN does not take into account the class distribution, being applied when no planar boundaries are needed [43]. In this case, the Euclidean distance of each test case was computed and the number of neighbors considered was three. After applying the DAGJK cross-validation, 81.7% and 73.2% of prediction efficiencies (Table 3) were obtained for EF and IF systems, respectively. Accordingly, KNN is comparable to SVM and LDA, and the best results are still obtained by MLP–ANN.

4. Conclusions

By using GC–MS and GC–FID six methylsterols have been identified and determined in subcutaneous fat of Iberian pigs. These compounds, which have been described for the first time in this type of samples, have been used as chemical descriptors to differentiate between pig fattening systems. The Mann–Whitney *U*-test has been applied to check statistical differences between the contents of the methylsterols in EF and IF systems. PR techniques have been used to obtain classification models. By applying BP–MLP–ANN a classification model was constructed using the six determined methylsterols with an overall classification performance of 91.7%.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.12.006.

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