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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.