Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography A, 1123 (2006) 82–91
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Characterization and quantification of the hydrocarbons fraction of the subcutaneous fresh fat of Iberian pig by off-line combination of high performance liquid chromatography and gas chromatography

A. Gamero-Pasadas [a], I. Viera Alcaide [a], J.J. Rios [a], E. Graciani Constante [a], I.M. Vicario [b], M. León-Camacho [a,*]

[a] *Department of Analysis, Instituto de la Grasa (C.S.I.C.), Av. Padre García Tejero, 4 41012 Seville, Spain*
[b] *Department of Nutrition and Food Science, Faculty of Pharmacy, University of Seville, Seville, Spain*

Received 6 March 2006; received in revised form 25 April 2006; accepted 28 April 2006
Available online 22 May 2006

Abstract

Hydrocarbons in fresh subcutaneous fat of Iberian pig have been analyzed by GC–MS after fractionation of the unsaponifiable fraction with a new off-line combination of HPLC and GC method. The new method proposed improves the recovery and simultaneous quantification of terpenic hydrocarbons in comparison to the traditional LC method. When necessary and for identification purposes, selective ion monitoring (SIM) was used as acquisition mode in GC–MS. To determine the position of the double bonds in the unsaturated hydrocarbon chain the dimethyl disulfide derivatives (DMDS) were obtained. To elucidate the structure of the branched 1-alkenes the hydrocarbon fraction was submitted to hydrogenation. Thirty-five compounds have been identified, including *n*-alkanes, *n*-alkenes, branched (*n*-1,*n*-2-dimethyl-1-alkenes) and terpenic hydrocarbons, being the most abundant *n*-alkenes and *n*-alkanes of even chain of *n*-C_{12}–*n*-C_{26}. Besides the hydrocarbons already described in bibliography, a new diterpenic hydrocarbon, ent-kaurene, have been identified for the first time. The compound reported as Neophytadiene by other authors, has been identified as a 20 atoms hydrocarbon with two double bonds, the 7,11,15-trimethyl-heptadeca-1,4-diene.

Keywords: Iberian pig; Subcutaneous fat; *n*-Alkanes; *n*-Alkenes; Branched hydrocarbons; Neophytadiene; Squalene; Kaurene

1. Introduction

Hydrocarbons are minor components of the unsaponifiable fraction of oils and fats. Most fats contain small quantities (0.1–1.0%) of saturated and unsaturated hydrocarbons, mainly *n*-alkanes from C_{10} to C_{35}, being the odd numbered carbon chains the most abundant [1]. The hydrocarbon profile is characteristic of each vegetable specie, for that reason it has been used for assessing authenticity of vegetable fats [2], e.g. olive oil varieties can be detected by means of their *n*-alkane profile [3].

Several authors have described *n*-alkanes, *n*-alkenes and branched hydrocarbons in ruminant animal tissues [4,5]. In the case of monogastric animals, like the Iberian pig, a fraction of linear saturated hydrocarbons has been characterize in the subcutaneous fat of fresh hams where the series from *n*-C_{14} up to *n*-C_{29}, excepting *n*-C_{23} and *n*-C_{28}, have been identified [6]. Also *n*-alkanes from *n*-C_{12} to *n*-C_{32} in the intramuscular fat of fresh hams have been reported [7], however hydrocarbons of low chain up to *n*-C_{12} have not been identified. Recent works have also identified branched hydrocarbons as neophytadiene and squalene, among others already identified in bovine tissues [5], in the intramuscular fat of fresh hams [8]. However the majority of the branch hydrocarbons remain unidentified due to their low levels, although it has been reported that they all have a methyl group in the linear carbon chain [8]. In addition, the *n*-alkanes and *n*-alkenes have also been identified in the intramuscular fat of dry-cured hams [9], concretely of *n*-C_{14} to *n*-C_{32}. In conclusion the fraction of *n*-alkenes is major than that of *n*-alkanes and the most abundant in both cases are those of short chain and even number of atoms of carbon. In the case of the *n*-alkenes odd, chain hydrocarbons have not been described. Finally, branched hydrocarbons have also been studied in the intramuscular fat of

* Corresponding author. Tel.: +34 95 4611550; fax: +34 95 4616790.
E-mail address: mleon@cica.es (M. León-Camacho).

0021-9673/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2006.04.081

dry-cured hams [10], however the majority of the compounds found remain still unidentified.

The hydrocarbon fraction is of great interest since it can be used as a key parameter to differentiate between the types of diet followed by the animals previous to slaughter. The traditional feeding of Iberian pigs is based on acorns and pasture. In acorns the hydrocarbon profile is composed by medium chain-alkanes (C_{29} and C_{27}), which means detection of about 80% of total hydrocarbons, while detection in case of other *n*-alkanes is less than 10% [11]. Nevertheless the situation appears confused in spite of the studies carried out by several authors on the subcutaneous and intramuscular lipid fraction of fresh and dry-cured products. This situation can be due to several factors: first the low levels in which these compounds are found, are usually below the limit of detection of the analytical methods. Hence, it is not feasible to characterize and quantify them simultaneously in the same fraction with the current analytical method employed. On the other hand the methods that allow the isolation of larger quantities of these compounds are long and tedious. For this reason the isolation of the unsaponifiable fraction of lipids by thin layer chromatography (TLC) and liquid chromatography (LC) are being replaced by HPLC methods [12].

The aim of the present study is to focus on the complete identification of the hydrocarbon fraction of fresh subcutaneous fat of Iberian pig. As a previous step to achieve this objective, it is necessary to improve the fractionation of the unsaponifiable fraction, with a reliable analytical method that allows both recovery and quantification of the hydrocarbons simultaneously. Thus an alternative analytical approach based on HPLC-GC off line fractionation of the unsaponifiable fraction is proposed.

2. Experimental

2.1. Reagents and standards

Hexane, fraction from petroleum, Multisolvent™ HPLC ACS grade supplied by Scharlau (Barcelona, Spain) was distilled through a fractionation column. Potassium hydroxide 85% pellets PA-ACS grade supplied by Panreac (Barcelona, Spain). Chloroform for analysis grade Merck (Darmstadt, Germany). The adsorbent used for column chromatography was silica gel 60 extra pure (70–230 mesh ASTM) from Merck Ref. 7754 (Darmstadt, Germany). Methyl disulfide (purum, >98%) Fluka Ref. 40221 (Seelze, Germany) was used for made the dimethyl disulfide derivatives. Platinum(IV) oxide hydrate (80% Pt) hydrogenation catalyst for synthesis was used, Merck Ref. 7346 (Darmstadt, Germany). All other materials were analytical grade. *n*-Eicosane (*n*-C20; Sigma Chemical Co., St. Louis, MO, USA) was used as an internal standard. *n*-Hexane (95%) code H389 and ethyl acetate code H346 both super purity solvent grade were supplied by ROMIL (Cambridge, U.K.) and were used as HPLC solvents.

2.2. Instrumentation

The unsaponifiable fraction was isolated by HPLC. 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 exit of the detector for to recovery of the hydrocarbons 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 hydrocarbons fraction was analysed in a VARIAN (Palo Alto, CA, USA) 3800 gas chromatograph equipped with a split/splitless injector and a flame ionisation 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 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.

The GC-ion-trap-MS experiments were performed using a FinniganTrace-GC2000 gas chromatograph coupled to a Polaris-Q ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA) equipped with an AS2000 autosampler operating in full scan mode from 50–600 amu at 1 scan s^{-1} and in selective ion monitoring (SIM) mode for identification purpose. The column used was a Zebron ZB-5 ms (Phenomenex, Torrance, CA, USA) fused silica capillary column (30 m long × 0.25 mm i.d × 0.25 μm film thickness). The GC conditions included helium as carrier gas at 1.0 mL min^{-1} in constant flow mode, a initial temperature at 114 °C, 4 °C min^{-1} to 310 °C, followed by an isothermal period of 11 min at the latter temperature, split injection mode was used, injection volume was 1 μL.

The MS operating conditions were the following: ion source and transfer line temperatures were 200 and 290 °C, respectively. The electron energy was 70 eV a resolution of one and the emission current 250 μA; dwell time and inter-channel delay was 0.08 and 0.02 s respectively. For GC-ion-trap-MS in SIM mode experiments, optimized parameter of buffer gas was set to 0.3 mL min^{-1} helium. Xcalibur version 1.4 software was used for data acquisition and processing of the results.

2.3. Samples and sample treatment

Eight samples of Iberian pig subcutaneous fat were used. The samples were obtained by heating in microwave oven according to the procedure described in the Spanish regulation [13]. Each sample was divided in four aliquots and the unsaponifiable