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Determination of androstenone in pig fat using packed column supercritical fluid chromatography–mass spectrometry

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Abstract

Packed column supercritical fluid chromatography (SFC) in combination with atmospheric pressure chemical ionisation mass spectrometry was applied to the analysis of androstenone in pig fat samples. Liquefied fat samples were dissolved in dichloromethane and analysed directly by SFC without any sample purification. Chromatographic separation was achieved with a density/pressure gradient using pure carbon dioxide as the mobile phase and the analysis resulted in a quantitation limit of 0.25 μg/g with 1 μl injection volume. Good agreement was found between the SFC method and time-resolved fluoroimmunoassay by the analysis of 15 boar back fat samples. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Animal fat; Androstenone; Steroids

1. Introduction

Raising of uncastrated male pigs (boars) is not practised in many countries due to the occasional presence of an offensive off-odour, "boar taint", in the meat. One of the main compounds associated with this problem is androstenone (5α-androst-16-en-3-one)[1], which is synthesised in the testes of boars, released into the blood and, because of its lipophilic properties, is taken up by adipose tissue and stored in the fat [2].

Several chromatographic methods have been developed for the measurement of androstenone, including gas chromatography (GC) with or without mass spectrometric (MS) detection [3–10], high-performance thin-layer chromatography [11], and high-performance liquid chromatography (HPLC) [12]. These analyses typically involve extraction procedures and further clean-up and derivatisation steps.

In recent years, supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) have been shown to be useful techniques with many desirable features for separating biomolecules such as steroids. Some SFC applications have been developed for the analysis of steroids using either capillary [13,14] or packed [15–17] columns. While most steroids are suitable for SFC, it appears that compounds of increasing polarity may cause unsatisfactory elution patterns [18]. The solubility of polar steroids can be improved in capillary SFC by

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chemical derivatisation [13]. In packed SFC, the chromatographic behaviour can be affected by adding polar modifiers in the mobile phase [15–20]. Polar interactions play a significant role on silica-based columns and the increased polarity of the modified mobile phase has a much higher impact on the solubility of polar steroids than on that of non-polar ones [18,21]. Steroids with only one or two polar groups can usually be eluted using pure CO_2, but steroids containing three or more polar groups may require high concentrations of polar modifier to elute [22]. In the case of non-polar steroids like androstenone, adequate chromatography can be expected with pure CO_2.

SFE has been successfully employed in the isolation of androstenone from fat matrix, with the final determination using GC [9,10]. The aim of the current study was to investigate whether fat-containing samples could be analysed directly by SFC, thus eliminating the need for sample purification. SFC was combined with mass spectrometry (SFC–MS) [23], which also eliminated the need for sample derivatisation. The SFC–MS results obtained from boar samples were compared with those of a time-resolved fluoroimmunoassay (TR-FIA)[24].

2. Experimental

2.1. Materials

The steroids 5α-androst-16-en-3-one, 5α-androstane-3,17-dione, 5α-androstan-3-one, 19-hydroxy-4-androstene-3,17-dione, and testosterone were obtained from Sigma (St. Louis, MO, USA). Stock solutions were prepared by diluting pure compounds in dichloromethane to a final concentration of 1 mg/ml.

2.2. Samples

Fat tissue samples ($n=15$) were collected from the neck region of Finnish boars and stored at −20°C. The samples were heated in a microwave oven and 0.8 g of melted fat from each sample was transferred to a volumetric flask. Internal standard (4 μg of 5α-androstan-3,17-dione) was added and the samples were dissolved into HPLC-grade dichloromethane (Rathburn Chemicals, Walkerburn, UK) to a final volume of 2.0 ml. The solutions were used as such in the chromatography. All analyses were performed at least in duplicate.

2.3. Supercritical fluid chromatography

The chromatographic separations were carried out with a Lee Scientific Series 600 supercritical fluid chromatograph (Dionex, Salt Lake City, UT, USA) using a 100×1 mm I.D. Deltabond Cyano or ODS C_{18} column (Keystone, State College, PA, USA) packed with 5 μm particles. SFC-grade CO_2 (Scott Specialty Gases, Plumsteadville, PA, USA) was used as a carrier fluid. Samples were injected via an electrically and pneumatically controlled Valco switching valve (Valco Instruments, Houston, TX, USA) with an internal loop size of 1 μl. Chromatographic separation was optimised using a flame ionisation detector (FID) held at 400°C. The micropacked column was connected to the FID using a fused-silica capillary restrictor (40 cm×25 μm I.D., Dionex). Optimum separation was achieved at a constant temperature of 100°C with a linear density gradient from 0.18 to 0.45 g/ml with a ramp of 0.012 g/ml/min, and from 0.45 to 0.69 g/ml with a ramp of 0.020 g/ml/min.

2.4. Mass spectrometry

The packed-column SFC was combined with a Finnigan MAT TSQ-700 triple-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) by installing the SFC restrictor capillary through the sample inlet of the Finnigan MAT atmospheric pressure chemical ionisation (APCI) interface as described by Manninen and Laakso [23]. APCI vaporiser temperature was kept at 425°C. Preheated sheath (138 kPa) and auxiliary (10 ml/min) gases were used to compensate for the cooling effect resulting from the rapid expansion of CO_2 from the restrictor outlet. The reactant ion solvent was introduced into the APCI source by leading the sheath gas (N_2) line through a solvent bottle containing methanol. The corona needle current was 5 μA. The MS was operated in a selected ion monitoring mode (SIM) recording the molecular ions ($[M+H]^+$) of the target analyte and the internal standard, i.e. m/z

273 and 289 for 5α-androst-16-en-3-one and 5α-androstane-3,17-dione, respectively. Positively charged ions were scanned with quadrupole 1 using a scan time of 0.2 s and a mass scan step of 0.4 Da.

3. Results and discussion

The applicability of a C_{18} column for the separation of androstenone was tested in preliminary work. Although this column retained androstenone moderately well, the peaks were too broad to be acceptable (data not shown). The use of a cyano column, and thus an increased polarity of stationary phase, resulted in only a small change of retention, but improved the peak shape significantly. Optimal chromatographic conditions were determined for the separation of androstenone from other compounds present in the fat matrix by testing different density gradients involving one to four steps at a constant temperature in the range of 90–140°C. Fused-silica capillary restrictors with inner diameters of 10, 15, and 25 μm and different lengths (30–60 cm) were tested to minimise the pressure drop along the system. The most acceptable peak shapes were obtained with 40 cm×25 μm I.D. fused-silica tubing.

Fig. 1 presents a SFC–FID chromatogram of a pig fat sample with 2.5 mg/g of added androstenone. The analysis was optimised for the separation of androstenone from the other, unidentified peaks in the analysis and the latter density gradient was targeted for the quick removal of the late-eluting endogenous material from the column. The limit of detection for androstenone with a signal-to-noise ratio (S/N) of 3:1 was only 54 μg/g, reflecting the weak FID response of androstenone.

Most HPLC and GC detectors can be used in SFC, but MS detection remains unmatched when both sensitivity and specificity are required. SFC–MS interfaces have traditionally been based on direct introduction of the mobile phase into the ionisation chamber of a MS instrument. Recently, there has been a growing interest in devices utilising atmospheric pressure ionisation (API), mainly in APCI mode [15,25–27]. The API systems are rugged with near universal response to most analytes, and more importantly, the performance of detectors that work at atmospheric pressure is not dependent on carrier

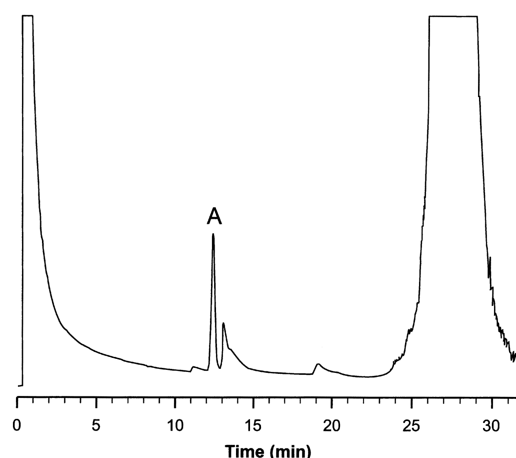

Fig. 1. SFC–FID chromatogram of a fat sample spiked with 2.5 mg/g of androstenone (peak A). The separation was accomplished on a Deltabond Cyano column (100×1 mm I.D.) packed with 5 μm particles. Column temperature was 100°C. Linear density gradient from 0.18 to 0.45 g/ml with a ramp of 0.012 g/ml/min, and from 0.45 to 0.69 g/ml with a ramp of 0.020 g/ml/min. Sample volume 1 μl.

fluid flow variations when density/pressure programming is used. Capillary SFC was recently combined with APCI-MS in our laboratory for the analysis of triacylglycerols in berry oils [23], and a similar setup was used in the current study. The interface could readily handle the flow from micropacked columns without the need for splitting the effluent. In addition, the cooling effect of the expanding carrier fluid could be compensated for without the use of extra heating devices. The nebulising gas was saturated with methanol to promote proton transfer reactions and to stabilise the ion current. In preliminary experiments, four different reactant ion solvents, namely methanol, isopropanol, water, and 0.5% ammonium hydroxide in methanol, were tested for the production of abundant protonated molecular ions. The most abundant $[M+H]^+$ ions were achieved with methanol.

The fragmentation of the analyte and the internal standard was obtained on full scan mass spectra of reference compounds (Fig. 2). Since APCI is a soft ionisation technique, it yields only little fragmentation and the spectrum is usually dominated by a protonated molecular ion. The loss of water yielding $[M+H-H_2O]^+$ ions was observed for both androstenone and the internal standard, as well as an adduct

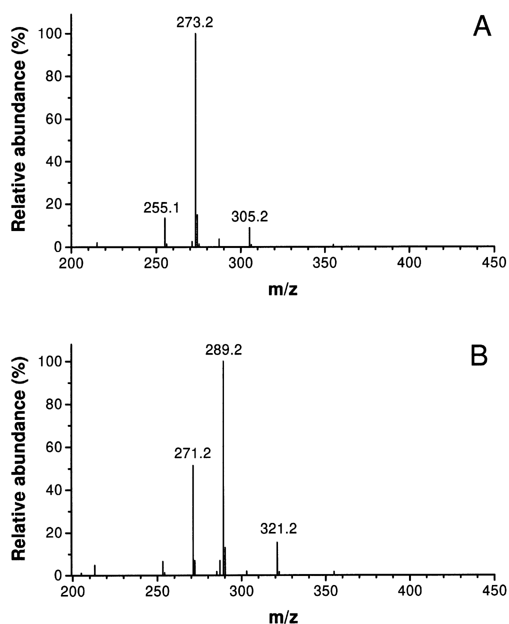

Fig. 2. The mass spectra of (A) 5α-androst-16-en-3-one and (B) 5α-androstane-3,17-dione using packed SFC–APCI-MS.

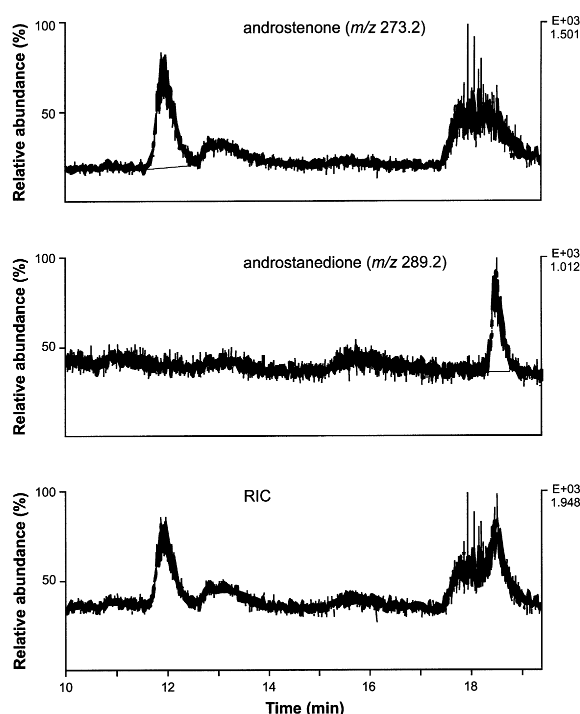

Fig. 3. Reconstructed ion chromatogram (RIC) for the analysis of a boar fat sample obtained by packed SFC with APCI-MS detection (no smoothing). Experimental conditions were the same as described for Fig. 1.

ion at $[M+33]^+$ which is presumably a proton-bound adduct between the analyte and methanol [15]. The internal standard was used in the analysis mainly to control the volume of the injection. In addition to androstanedione, also 5α-androstan-3-one (*m/z* 275), 19-hydroxy-4-androstene-3,17-dione (*m/z* 303), and testosterone (*m/z* 289) were considered as possible candidates for this use. However, the column could not resolve androstanone from androstenone and the two last-mentioned compounds were more strongly retained by the column and eluted at the middle of the large peak at the end of the run.

A reconstructed ion chromatogram (RIC) of an analysis of boar sample is presented in Fig. 3. The MS-interface installation did not affect the chromatographic resolution, and the elution of androstenone and androstanedione was reproducible with mean retention times of 11.97 min (C.V.=1.6%) and 18.55 min (C.V.=0.9%), respectively ($n=10$). The limit of quantification for androstenone was 0.25 μg/g (C.V.=6.6%, $n=3$), demonstrating a clearly superior sensitivity compared to FID. The calibration curve ($y=0.377x+0.032$) was linear over the range of 0.1–10 μg/ml, corresponding to androstenone concentrations of 0.25–25 μg/g in fat (standard error 0.139, $r=0.999$, $n=20$). Some experiments were also conducted with an external 10 μl sample loop, and the results indicated that at least a ten times larger injection volume could be used without a loss of chromatographic separation. Thus, the method sensitivity could still be improved by simply injecting a larger sample volume to the system. The method was found to have sufficient precision and accuracy. The mean recovery of spiked gilt fat samples was 96.7% (Table 1) and repeated analyses of fat samples containing 2.09 and 0.32 μg/g of

Table 1
Accuracy of the androstenone SFC–APCI-MS analysis

Added (μg/g)	Measured (μg/g)	Recovery (%)	*n*
5.0	4.45	90	4
2.5	2.35	94	4
0.5	0.53	106	2

androstenone yielded coefficients of variation of 3.5% and 5.7%, respectively ($n=5$).

The performance of a time-resolved fluoroimmunoassay [24] was confirmed by the developed SFC method. Adipose tissue samples from 15 boars were selected for the SFC analysis on the basis of results obtained by the TR-FIA. Concentrations up to 4.5 μg/g of androstenone were observed with two samples falling below the method sensitivity. The results from SFC and TR-FIA agreed well up to 4 μg/g of androstenone with a correlation coefficient of 0.994 ($p<0.001$) (Fig. 4). Statistical evaluation gave the regression equation $y=0.95x - 0.127$ (standard error 0.11) and the standard deviations for the slope and the intercept were 0.030 and 0.043, respectively. The slope did not deviate significantly from 1 (95% confidence interval 0.95±0.07), but the immunochemical method gave slightly higher results (95% confidence interval for the intercept −0.127±0.094). The Deming regression, which may be more preferable in method comparison studies [28], also gave similar results ($y=0.96x-0.136$; $r=0.994$). An extra dilution was needed in the immunochemical analysis for samples with concentrations above the calibration range (>4.0 μg/g). This may lead to slight deviations in the results [29], which is presumably due to the matrix effects in the assay. However, the overall agreement between the results of the two methods was good, which is in accordance with previous studies comparing chromatographic methods and immunochemical androstenone analysis [9,12,29].

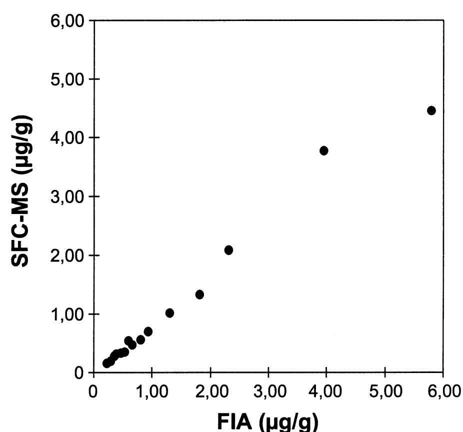

Fig. 4. Comparison of the results from androstenone determination in boar fat samples, using SFC–MS and TR-FIA ($n=15$). See text for regression data.

4. Conclusions

This study demonstrates the potential of packed column SFC–APCI-MS for the analysis of androstenone from porcine fat samples. The sample preparation is extremely simple, requiring only the dissolving of the fat sample in adequate solvent for chromatography. The minimised sample handling and the high specificity provided by mass spectrometric detection result in a very accurate and precise method that is useful in the evaluation of other analytical techniques, e.g., immunochemical methods.

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