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QUANTITATIVE DETERMINATION OF ANDROSTENONE IN PIG ADIPOSE TISSUE

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SUMMARY

A simple, cheap and rapid method for the quantitative determination of the boar taint substance, 5α-androst-16-en-3-one, in pig adipose tissue is described. After saponification of the fat the androstenone is extracted, derivatised with *o*-(pentafluorobenzyl)hydroxylamine hydrochloride in pyridine and analysed by fused-silica open-tubular capillary gas chromatography with electron-capture detection.

INTRODUCTION

For meat production, the rearing of entire male pigs (boars), instead of the castrated animals (barrows) is very economically advantagous[1,2]: during development, boars use their food more efficiently, and so grow faster and produce leaner carcasses than barrows or gilts. However, the commercialization of boar carcasses is hampered by the so-called boar taint, a strong unpleasant cooking odour, exhibited by some (5–20%) of the boar meats. The unpleasant odour is associated with the fatty tissue and, more precisely, with the non-saponifiable fraction[3,4]. Sink[5] postulated that the odour was due to C_{19}-Δ_{16} steroids and that they may function as sex pheromones in pigs. In 1968 Patterson[6] isolated a 16-unsaturated steroid: 5α-androst-16-en-3-one (androstenone) and claimed it as the principal component responsible for boar taint. Since then several correlations (r = 0.48–0.76) between the androstenone content of the fatty tissue and boar taint have been obtained[7–10]. However, other malodorous compounds, such as indole[11,12], skatole[12–14] and volatile aldehydes[15] have also come under suspicion of contributing to boar taint.

The idea of selecting tainted boar carcasses by determining the androstenone content of the boar fat has encouraged research on reliable methods for androstenone determination. Chromatographic procedures, involving numerous extraction and purification steps followed by gas chromatography (GC)[16–18] or gas chromatography–mass spectrometry (GC–MS)[19] are very time-consuming. The separation of small amounts of androstenone (0.02–5 ppm) from relatively large amounts of fat requires plenty of labour and of organic solvents. Radioimmunological analysis (RIA), as developed by Andresen[20,21] and Claus[22], is more suitable

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for routine measurements, but the use of radioactive isotopes strongly reduces the extension of this technique for systematic checking of all boar carcasses. The non-isotopic immunological detection of androstenone in pork fat would permit simple and low-cost screening of tainted carcasses. However, the enzyme-linked immunosorbent assay (ELISA) method as developed by Storm (Intervet)[23], is not yet commercially available.

The present paper presents a relatively fast, simple and sensitive GC method for the determination of androstenone in pig fat. After a simplified extraction procedure, androstenone is derivatized for electron-capture detection (ECD) and analysed by capillary GC.

EXPERIMENTAL

Reagents and reference compounds

Androstenone [5α-androst-16-en-3-one; Δ16-(5α)androsten-3-one] and androstanone (5α-androstan-3-one) were obtained from Sigma (St. Louis, MO, U.S.A.). Florox reagent (*o*-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA–HCl; 2.5 mg/ml in E.C. grade pyridine) was purchased from Pierce (Rockford, IL, U.S.A.). All other reagents used were of analytical reagent grade and used without further purification.

Solutions

A stock standard solution of androstenone and androstanone (internal standard) was prepared by dissolving 1 mg of androstenone or androstanone in 1 ml of cyclohexane. This standard stock solution was diluted 25-fold to give working solutions of 40 ng/μl cyclohexane. The addition of 20 μl of working solution to 400 mg of fat (the amount used for analysis) is equivalent to a concentration of 2 mg/kg (2 ppm). From the working solution of androstenone dilutions equivalent to 1.5, 1 and 0.5 ppm were prepared.

Chromatographic conditions

The gas chromatograph used was a Varian 3700, equipped with a capillary injection system and an electron-capture detector. Integration was performed with a Varian Vista 401 chromatographic data system. Chromatograms were plotted with baselines from raw data stored on floppy disc. The electrometer settings on the 3700 were: range, 10; attenuation, 8. The chromatograms were attenuated with the integrator. The column was a fused-silica capillary (50 m × 0.23 mm I.D.) coated with CP Sil 5 (OV-1, SE-30 analogue) from Chrompack (Middelburg, The Netherlands).

Hydrogen was used as carrier gas at a flow-rate of 1 ml/min. The splitting ratio was 1:25. Nitrogen was used as make-up gas for the detector at 30 ml/min.

The column, injector and detector temperatures were 270°C, 310°C and 330°C, respectively. The retention times of the *syn* and *anti* forms of the PFBHA derivatives of the steroids were: androstenone, 8.4 and 8.8 min; androstanone, 9.2 and 9.6 min.

Apparatus

The following apparatus was used: a centrifuge, a reacti-term heating module (Pierce, Rockford, IL, U.S.A.), a nitrogen jet evaporator, made in our laboratory[24],

round-bottomed extraction tubes (15 ml, 10 cm × 15 mm I.D.; 8 ml, 9 cm × 10 mm I.D.) and conical reaction tubes (5 ml, 7 cm × 10 mm I.D.).

During the course of this work a handy and an inexpensive shaking apparatus (see Fig. 1) was constructed from a commercial electric saw with adjustable speed (*e.g.* Black & Decker DN 39). The saw (blade) was replaced by a box, capable of taking a rack of eight round-bottomed extraction tubes. The electric saw was mounted on a wooden support, standing on four rubber blocks that damped the vibrations. Shaking is carried out at a moderate speed (2–3 on the Black & Decker scale).

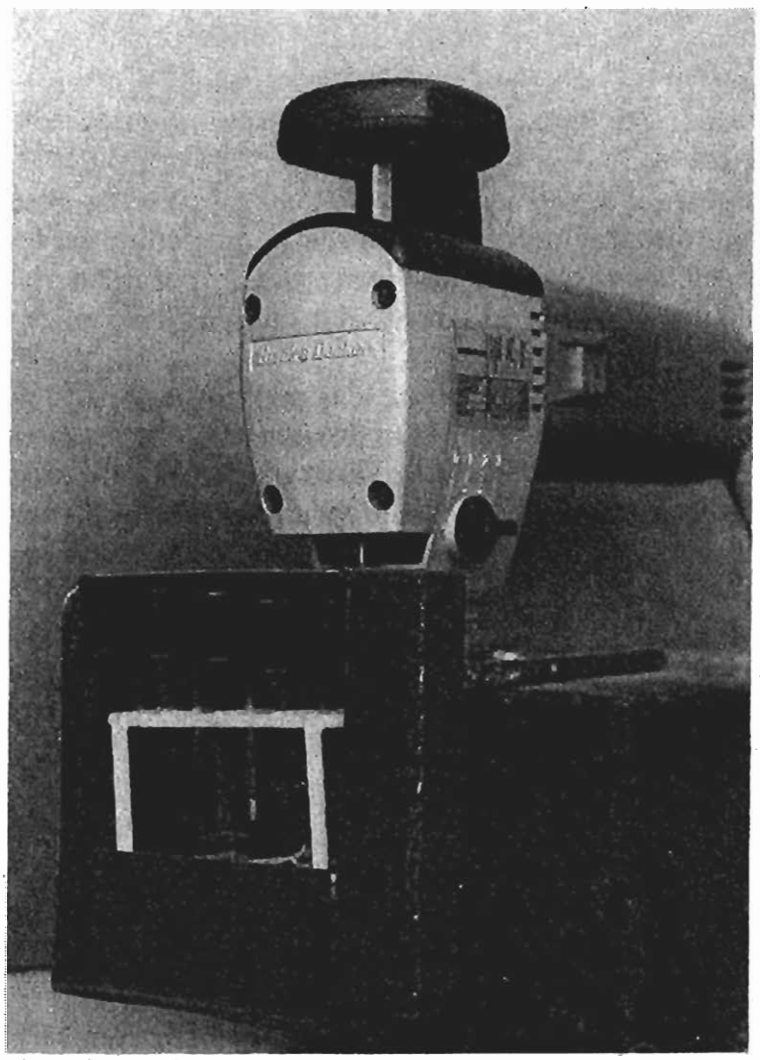


Fig. 1. Shaking apparatus, constructed from a commercial electric saw.

Analytical procedure

A 0.4-g sample (*e.g.* fat) was placed into a 15-ml extraction tube with screw stopper, and 1.4 ml of toluene, 2 ml of potassium hydroxide solution (10% in methanol) and 20 μl of internal standard solution (androstanone) were added. The mixture was shaken and heated at 80°C for 1 h in a reacti-term heating module. After cooling, 2.5 ml of methanol, 2 ml of distilled water and 5 ml of light petroleum were added. The mixture was shaken for 30 s on our shaking device (Fig. 1). Phases were separated

by centrifugation at 2000 rpm (500 *g*) for 10 min. The clear upper phase was transferred to a 8-ml silanized extraction tube. The volume was reduced to *ca.* 1 ml under a jet of nitrogen. The contents of the tube were transferred to conical 5-ml reaction tubes. The solvent was evaporated to dryness under a jet of nitrogen, 5 μl of Florox reagent were added, and the tube was heated at 100°C for 1.5 h in a reacti-term heating module. After cooling, 40 μl of cyclohexane were added and the tube was heated again for 5 min at 100°C, before 1 μl of the contents was injected into the gas chromatograph.

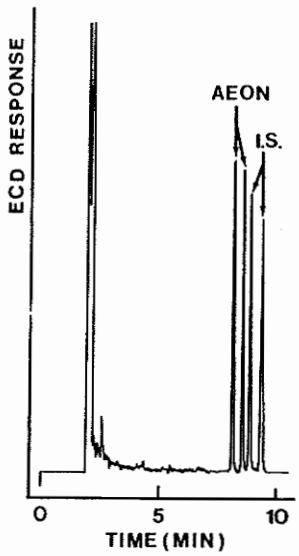
RESULTS AND DISCUSSION

Derivatization of androstenone

The GC determination of androstenone in pig adipose tissue, using packed columns and flame ionization detection (FID), has been described previously[16–18]. However, FID is non-specific, and a laborious clean-up procedure involving both column and thin-layer chromatography was needed before the sample could be injected. The derivatization of androstenone for ECD and the separation of the derivatives by capillary GC would enhance both the specificity and the sensitivity of detection and reduce the complexity of the clean-up.

The usual derivatization agents for ketosteroids, such as perfluoroanhydrides, react with a hydroxyl function, but this is not present in androstenone. The classical reagents for preparing derivatives from ketones include hydroxylamines (formation of oximes), semicarbazides (yielding semicarbazones) and phenylhydrazones (formation of hydrazones). In 1975, *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA-HCl) was introduced by Koshy *et al.*[25] and Nambara *et al.*[26] as a sensitive derivatizing agent for the electron-capture gas–liquid chromatographic (GLC) analysis of ketosteroids. A ready-to-use reagent, containing 2.5 mg/ml PFBHA-HCl in E.C. grade pyridine (Florox reagent) was commercialized by Pierce. This reagent was tested and it was found that androstenone (and also the internal standard, androstanone) is readily derivatized by heating with 5 μl of Florox reagent at 65°C for 1.5 h. After dilution of the reaction mixture with cyclohexane (40 μl), the derivatives may be injected on a splitter injector without further purification. Fig. 2 shows a chromatogram of a standard solution of androstenone, equivalent to a concentration of 2 ppm in adipose tissue (400 mg). For each steroid two peaks with an identical peak area were found. Although the nature of the two peaks was not investigated, they probably correspond to the *syn* and *anti* forms of the PFBHA derivatives, as described by Koshy *et al.*[25]. In this study, the two geometric isomers are baseline-separated by the high separation power of the capillary column.

The reaction rate of androstenone with PFBHA was studied as a function of temperature by using a standard solution, equivalent to 2 ppm. During this study an appropriate amount of mirex (perchloropentacyclohexane) was added to the mixture as an additional internal standard (retention time *ca.* 5.7 min) because of its constant peak area. The ratio of the peak areas of the androstenone derivatives to mirex was calculated. Fig. 3 shows the derivatization yield *versus* time curves for androstenone at different temperatures. Derivatization is complete after heating for 1.5 h at 100°C. An analogous result was found for androstanone. In comparison with the procedure described by Pierce[27], a higher temperature and a longer reaction time for the quan-



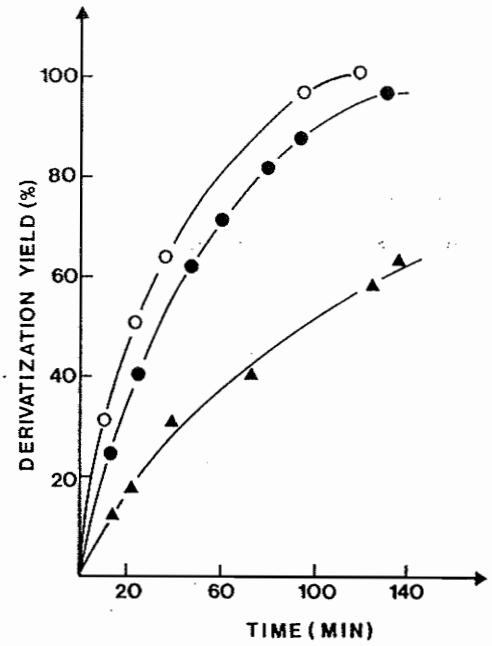


Fig. 2. Gas chromatogram of a standard solution of androstenone (AEON) and androstanone (I.S.), equivalent to a concentration of 2 ppm in adipose tissue.

Fig. 3. Derivatization yield of androstenone as a function of time at different temperatures: ▲ = 25°C; ● = 65°C; ○ = 100°C.

titative derivatization of androstenone is needed. After dilution of the reaction mixture with cyclohexane (40 μl), the solutions of the derivatives are stable at room temperature for weeks.

Calibration graph

The relative response factor of androstenone *versus* the internal standard androstanone was determined by using standard solutions. Since the only difference between androstenone and androstanone is a double bond at C-16 the relative response factors are equal. A linear calibration graph (Fig. 4) was obtained by plotting the ratio of the peak area of the first androstenone peak to the peak area of the first internal standard peak. The curve illustrates the linearity of the method over the concentration range studied.

Extraction and clean-up

In the methods previously described, androstenone is separated from the main amount of fat by high vacuum distillation[6,18], precipitation of the fat in organic solvents by cooling[20] or solvent partition[16,17]. A clean-up procedure using the volatility of androstenone may be very selective, but requires much labour and is not suitable for routine analysis. Precipitation of fat by cooling was tested, but we found that small amounts of fat, not removed by precipitation, inhibit the derivatization with PFBHA. Solvent partition, after saponification of the fat, results in complete removal of fat but a large amount of solvent is needed to prevent the formation of emulsions (*e.g.* 60 ml of solvent per gram of sample[17]).

In 1976, Kaufman *et al.*[16] described an extraction procedure that used a qua-

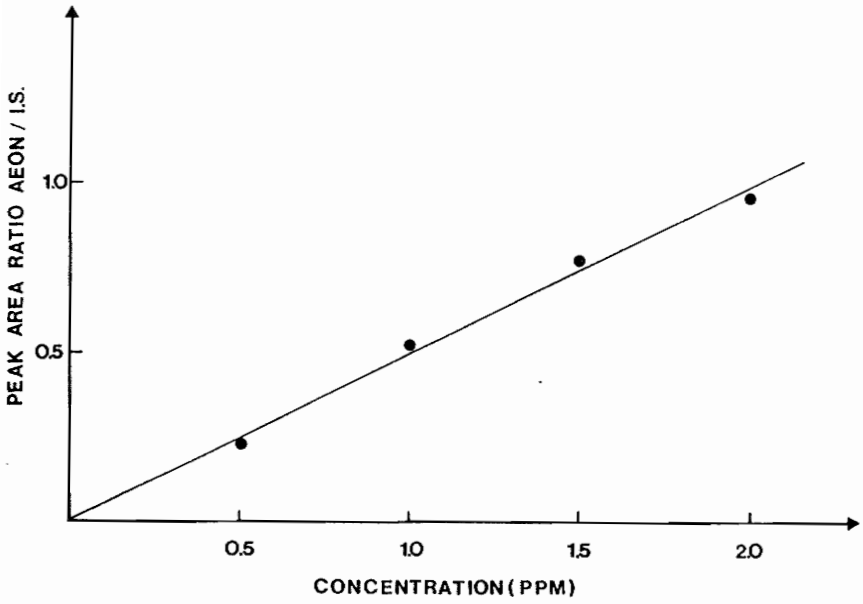


Fig. 4. Calibration graph for androstenone (AEON).

ternary mixture of water–methanol–benzene–light petroleum (or hexane). With this method androstenone was separated from the saponified fat without formation of emulsions and at a ratio of 30 ml of solvent per gram of fat. This procedure was tested on a boar fat, and the chromatogram, obtained after derivatization of the extract, showed clearly the presence of androstenone. In our procedure benzene was exchanged for the less toxic toluene. Since ECD is more sensitive than FID, the amount of sample and solvent could be scaled down (7.5 times) so that the total procedure (saponification and extraction) could be carried out in one test-tube.

Androstenone (resp. androstanone) was extracted for 93 ± 2.5% (resp. 98 ± 3.5%) in the upper phase of a single equilibration of 1.4 ml of toluene (11%), 4.5 ml of methanol (35%), 2 ml of distilled water (15%) and 5 ml of light petroleum (39%). The relative response factor of androstenone *versus* androstanone, after extraction, was calculated as 0.95.

Fig. 5 shows the chromatograms of three adipose tissues, extracted as described above. They illustrate that the clean-up provided by the modified Kaufman procedure[16] meets the requirements of the capillary GC–ECD determination of androstenone in the range 0.08–2.6 ppm. The PFB-oxime derivatives (*syn* and *anti*) are baseline-separated from each other. The androstenone-PFB oxime derivatives are also well separated from the internal standard derivatives and from derivatives originating from the matrix.

Recovery

The recovery of androstenone was determined using adipose tissue from two gilts, at six different concentrations, ranging from 0.125 ppm to 2 ppm. Standard and internal standard solutions (20 μl) were added to the saponification mixture before heating, and the procedure was carried out as described above.

Table I shows that 100% of the added androstenone is recovered over the

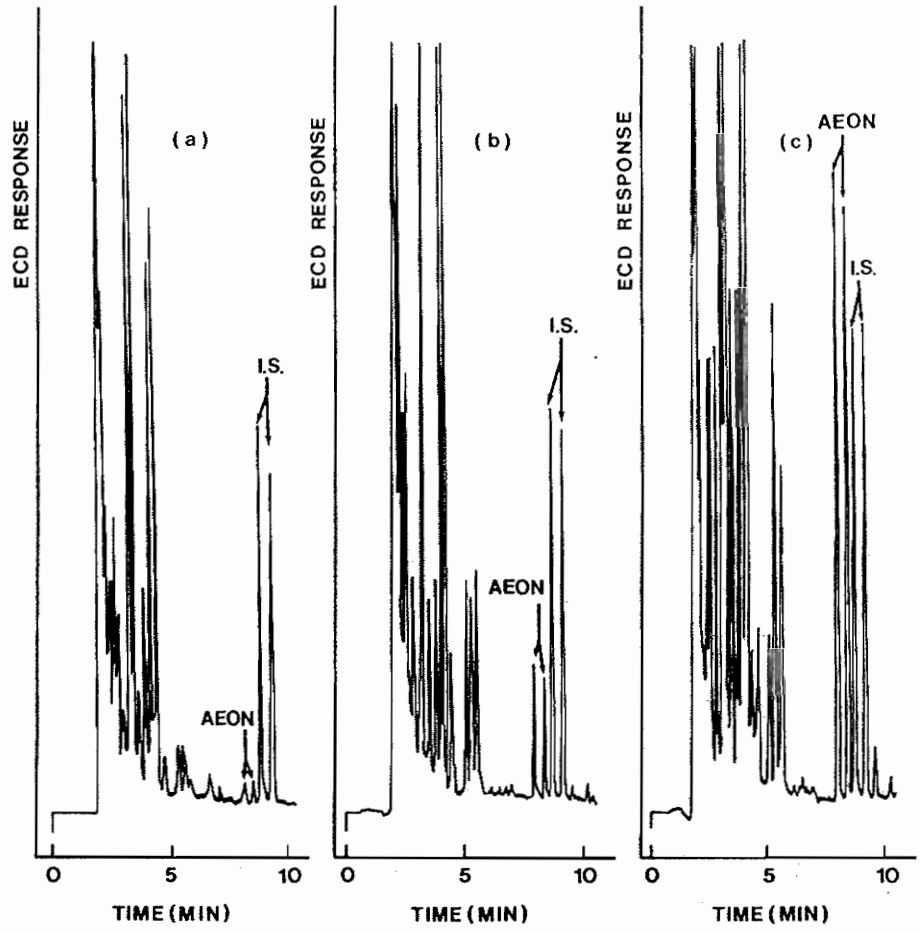


Fig. 5. Gas chromatograms of adipose tissue from (a) female pig (0.08 ppm AEON), (b) boar (0.66 ppm AEON), (c) boar (2.6 ppm AEON). Androstanone (I.S.) equivalent to 2 ppm).

TABLE I

RECOVERY OF ANDROSTENONE FROM PIG ADIPOSE TISSUE

Three determinations were carried out on each sample.

Androstenone concentration (mg/kg)			*Recovery (% ± S.D.)*
Before addition	*Added*	*After addition*	
0.073 ± 0.011	0.125	0.203 ± 0.012	103 ± 14
0.105 ± 0.008	0.125	0.230 ± 0.011	100 ± 17
0.073 ± 0.011	0.25	0.315 ± 0.019	100 ± 12
0.105 ± 0.008	0.25	0.356 ± 0.019	100 ± 10
0.073 ± 0.011	0.5	0.614 ± 0.074	107 ± 15
0.105 ± 0.008	0.5	0.600 ± 0.053	99 ± 11
0.073 ± 0.011	1.0	1.050 ± 0.025	98 ± 3
0.105 ± 0.008	1.0	1.064 ± 0.059	96 ± 6
0.073 ± 0.011	1.5	1.643 ± 0.059	104 ± 4
0.105 ± 0.008	1.5	1.688 ± 0.015	105 ± 1
0.073 ± 0.011	2.0	1.968 ± 0.099	95 ± 5
0.105 ± 0.008	2.0	2.102 ± 0.048	100 ± 2
Mean ± S.D.			100 ± 3.7

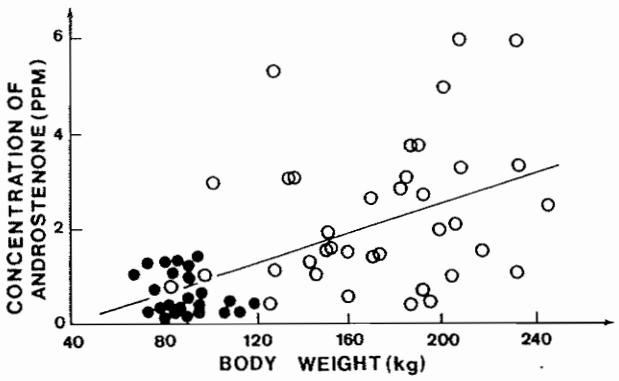


Fig. 6. Plot of androstenone concentration *versus* body weight of 82 Belgian boars: ● = younger boars; ○ = older boars ($y = 0.016\,x - 0.677$; $r = 0.633$).

whole concentration range investigated. Thus the method described is suitable for the accurate determination of androstenone over the whole concentration range needed for the evaluation of boar taint.

Androstenone concentrations found in some pigs

Using the method described above, the androstenone concentration in adipose tissue of 82 boars, 39 sows and 16 castrates was determined. The older boars (weight more than 100 kg) were imported from Germany. The younger boars (weight less than 100 kg), the sows and the castrates were of Belgian origin. During the analysis of these series no difficulties with the procedure (*e.g.* formation of emulsions) were observed. In sows and castrates low androstenone values were found [mean androstenone value of 0.04 ± 0.13 ppm (sows) and 0.11 ± 0.22 ppm (castrates)]. The results of the analysis of boar fats are shown in Fig. 6 as a plot of the androstenone concentration *versus* body weight. The correlation found ($r = 0.63$) is of the same magnitude as most correlations reported for boar taint *versus* androstenone concentration[7–10]. Table II summarizes the results of the analysis of boar fats. As expected, younger boars show a lower mean androstenone concentration than the older ones. However, even 47% of the younger boars scored androstenone concentrations higher than 0.43 ppm. According to Otto and Behm[7] the probability of boar taint is high above this lower limit. The results indicate that, if androstenone is the major component responsible for boar taint, the boar taint problem in Belgian boars should not be underestimated.

TABLE II

ANDROSTENONE VALUES OF SOME BOARS

n = Number of samples analysed.

Weight (kg) mean ± S.D. (n)	*Androstenone concentration (mg/kg)*		
	Mean ± S.D.	*Minimum*	*Maximum*
86 ± 12 (40)	0.6 ± 0.4	0.08	1.4
170 ± 42 (42)	2.2 ± 1.5	0.36	5.9

TABLE III

COMPARISON OF OUR METHOD WITH PREVIOUS CHROMATOGRAPHIC METHODS

CC = Column chromatography; TLC = thin-layer chromatography.

Ref.	*Method*	*Clean-up*	*Sample size (g)*	*Amount of solvent (ml)*
16	GLC–FID	TLC	3	benzene (3), methanol (45), hexane (70)
17	GLC–FID	CC–TLC	5	methylene chloride (125), ethyl acetate (130), cyclohexane (200)
18	GLC–FID	TLC	50	chloroform (65), methanol (15), diethyl ether (65)
19	GC–MS	TLC	2	ethanol (15), hexane (30)
This work	CGC–ECD	Extr.	0.4	toluene (1.4), methanol (4.5), light petroleum (5)

Comparison with previously published methods

Table III compares our method with methods previously described for analysis of androstenone in pig adipose tissue. The comparison is based on the kind of chromatographic method, the clean-up procedure, the amount of sample and the organic solvent used.

The high sensitivity of ECD for pentafluoro-oxime derivatives allows the fast and quantitative determination of androstenone over the whole concentration range needed (0.08–6 ppm), while permitting a smaller sample to be used. Moreover, the amount of organic solvent used is at least 10 times smaller than in the other chromatographic methods. In the future, the use of short narrow-bore fused-silica open tubular columns (*e.g.* 10 m × 0.1 mm I.D.) will permit the time of the chromatographic run to be shortened.

CONCLUSIONS

The method described here allows an accurate determination of androstenone in pig adipose tissues over the whole concentration range needed for boar taint evaluation (0.1–6 ppm AEON). In comparison with existing chromatographic methods, the analysis on a fused-silica open-tubular column with ECD enhances the simplicity and speed, permits smaller sample sizes, and reduces the use of expensive solvents.

The method was designed for large-scale laboratory research on the relationship between boar taint and androstenone concentration. It may also be useful in the evaluation of new and fast (immunological) methods. The immunological methods are superior in analysis capacity and speed but are prone to cross-reactions, leading to less accurate results.

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