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# QUANTITATIVE DETERMINATION OF THE BOAR TAINT SUBSTANCE 5α-ANDROST-16-EN-3-ONE IN FAT

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## SUMMARY

A method for quantitative estimation of the boar taint substance 5α-androst-16-en-3-one (androstenone) in adipose tissue is described, consisting of the following steps: 1. alkaline saponification and extraction from 70% aq. methanol by n-hexane, 2. t.l.c., 3. g.l.c.

Accuracy of the estimation was confirmed by two different g.l.c. conditions, by I.R. spectrum of the isolated androstenone and by measuring the content of androstenone after addition of the authentic substance to fat of a castrated animal. Regression analysis gave the function $y = 0.97x + 0.06$. Coefficients of variation were less than 10%, using homogeneous samples of adipose tissue containing more than 0.2 μg androstenone per g. Volatility of androstenone as a source of errors was investigated in detail.

The method permitted determination of androstenone content in adipose tissue of sows and castrated male pigs (0.02–0.06 μg/g). In boars (age 10–12 months) 0.08–2.45 μg/g (mean 0.85 μg/g) were found. A level of 0.1 μg androstenone per g adipose tissue is suggested as acceptable when the meat is provided for human consumption.

## INTRODUCTION

Fat and fatty meat of mature boars often emit an unpleasant smell on being cooked, which excludes utilization of such meat for human consumption. The odour is caused by the content of non-polar steroids, androst-16-enes with only one single oxygen function in position 3, which are formed in the testes and partially stored in adipose tissue. A detailed review about occurrence, synthesis, metabolism and physiological role of 16-unsaturated androstenes was given by Gower[1].

The principal substance responsible for boar taint in fat was found [2] to be 5α-androst-16-en-3-one (androstenone)* and in addition in one case 5α-androst-16-en-3α-ol [3]. There is a good correlation between androstenone content and boar odour [4].

For a quantitative estimation of androstenone in fat, the low content requires a relatively high expenditure of labour for separating large amounts of fat. Claus[5, 6] was successful with two column chromatographic separations on silica, t.l.c. and g.l.c. Patterson[2] and Fuchs[4] used volatility of androst-16-enes to separate them from the main amount of fat by high-vacuum distillation. A saponification step and purification by t.l.c. preceded the determination by g.l.c. [4]. Recently, Claus[7] and Andresen[8] described radioimmunoassays for androstenone in porcine adipose tissue. Steps of extraction and distribution [7] or precipitation of fat in organic solvents by cooling [8] precede the reaction with the antibodies.

The present paper describes another method, including removal of fat by saponification and extraction without formation of emulsions, so that after t.l.c. the androstenone can be determined by g.l.c.

## EXPERIMENTAL

The solvents used were chemically pure and were distilled before use. Chloroform was distilled over potassium carbonate, benzene was dried over calcium hydride and distilled. Ether was dried over sodium and distilled. Melting points are uncorrected. Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3375, using the toluene system containing 0.5% PPO and 0.03% dimethyl-POPOP.

*Synthesis of reference substances. 5α-Androst-16-en-3α-ol* was synthesized from 3α-acetoxy-5α-androstan-17-one by reaction of the tosylhydrazone with methyl lithium [9, 10]: 500 mg 3α-acetoxy-5α-androstan-17-one and 400 mg p-toluene-sulfonylhydrazide in 2.5 ml methanol refluxed 3 h and cooled gave the tosylhydrazone in 90% yield.

A solution of 500 mg of the tosylhydrazone in 20 ml anhydrous ether was stirred under an atmosphere of dry argon at room temperature, and within 4 h a solution of methyl lithium was added, obtained from 168 mg Li and 0.85 ml $CH_3I$ in 15 ml anhydrous ether under argon. After stirring for an additional 2 h water was added and the steroid extracted with ether. The

* Abbreviation: androstenone = 5α-androst-16-en-3-one.

ether extract was washed with 1 M NaOH followed by water, dried over sodium sulphate, evaporated and chromatographed on alumina. Yield 50% 5α-androst-16-en-3α-ol, m.p. 144–146°C (from ethanol) (Lit. [11]) m.p. 143.5–144°C).

*5α-Androst-16-en-3-one* was synthesized from the 3-hydroxy compound by oxidation with $CrO_3$ in acetone [12]. Yield 85%, m.p. 145–146.5°C (from ethanol) (Lit. [13] m.p. 140–141°C).

Androstenone was labelled with $^3H$ according to Wilzbach[14] in the Institute for Applied Radioactivity, Leipzig. The substance, freed from labile $^3H$, was purified by t.l.c. on silica gel $GF_{254}$ (Merck), solvent system benzene–ethyl acetate 8:2. Furthermore, for removal of $^3H$, labile under conditions of saponification, 20 μg androstenone-[$^3H$] were refluxed 30 min with 15 ml 4% methanolic KOH (w/v) and 10 ml benzene, followed by addition of water and extraction with hexane. This process was repeated three times. The material was then purified by t.l.c. as described above. The radioactivity decreased during the first treatment to 70% and then remained nearly constant. On the assumption that no loss of substance took place but only exchange of $^3H$, the S.A. of the androstenone-[$^3H$] (G) was calculated to be 4.5 μCi/μg.

*Work-up of samples. Collecting samples.* The samples of adipose tissue taken from the side area of German Landrace pigs, were frozen and stored at −18°C.

*Extraction.* 3 g adipose tissue were cut into small pieces, 0.01 μCi (0.002 μg) androstenone-[$^3H$] added and refluxed for 15 min three times with ethyl acetate (3 × 20 ml). The pooled extracts were evaporated on a rotary evaporator and dried after addition of a little ethanol to give 2.0–2.8 g fat.

*Saponification.* To the fat obtained or directly to 3 g chopped tissue, 10 ml benzene and 15 ml 10% methanolic KOH (w/v) were added. The mixture was boiled under reflux for 1 h, then 20 ml methanol and 15 ml water were added and the mixture extracted with 30 ml hexane (or light petroleum, b.p. 30–50°C). No emulsions were formed even after vigorous shaking. The lower phase was extracted twice more with 20 ml hexane. Sometimes a third, middle phase appeared. After removing the lower phase, this third phase disappeared by addition of some 70% aq. methanol. The pooled upper phases were washed with 10 ml 70% aq. methanol followed by 10 ml water, dried over sodium sulphate and evaporated *in vacuo* at 30°C to give 3–12 mg unsaponifiable neutral substance.

*Adsorption chromatography.* The residue of saponification was dissolved in 0.2 ml hexane and 1 drop benzene and applied to a thin-layer plate as a 10 cm. band. Adsorbent: Kieselgel $GF_{254}$ (Merck), 200 × 200 × 0.5 mm, solvent system: benzene–ethyl acetate (8:2 v/v). A test mixture containing androstenone was spotted at the plate 2 cm. from the edges and, after running, detected by spraying this area with iodine in hexane. U.V. absorbing zones were marked under an U.V. lamp. In front of the androstenone ($R_F$ 0.58) in the residue of saponification an U.V. positive zone was always found, a second zone behind it in most cases. The androstenone was sucked into a small sintered-glass filter and eluted with 4 ml chloroform–methanol (19:1 v/v) into a flask containing 2–5 μg of the standard cholestane for g.l.c. After evaporation *in vacuo* and dissolving in 4 ml hexane, an aliquot (10%) was assayed for $^3H$-activity and the remaining solution evaporated *in vacuo*, transferred with hexane–chloroform (9:1 v/v) to small tubes, evaporated to dryness under a current of nitrogen and *in vacuo* (25 Torr, 30°C) and used for g.l.c.

At the beginning of our experiments, column chromatography was used instead of t.l.c. The residue of saponification was dissolved in 0.5 ml benzene–hexane (1:1 v/v) and chromatographed on silica gel (2.5 g, Merck 0.2–0.5 mm, column 8 × 120 mm). Elution with 1. benzene–hexane (1:1, 50 ml), 2. benzene (6.5 ml) and 3. benzene (35 ml) gave the androstenone in fraction 3, which was used for g.l.c. Recovery of the $^3H$-standard 75–85%.

*Gas–liquid chromatography.* Aliquots of 10–50% of the chromatographically separated androstenone fraction were analyzed, dissolved in chloroform, in a Varian Aerograph 1520 B, equipped with a flame ionisation detector, 3% QF 1 on Gaschrom Q (80–100 mesh). Glass column 400 × 0.3 cm., temperature 205°C, injector 245°C, carrier gas argon, 4 atm. Retention time 20.2 min, standard cholestane 24.8 min. Quantification was carried out by planimetry of peak areas.

Some samples were additionally investigated by g.l.c. at 2% XE 60 on Gaschrom Q (80–100 mesh). Glass column 200 × 0.3 cm., temperature 188°C, argon 1.5 atm., retention times androstenone 8.7 min, cholestane 18.5 min.

## RESULTS

(1) *Experiments on separation by volatility.* When we searched for a method for quantitative determination of androstenone in fat, at first we tried to use the volatility of androst-16-enes with steam for their separation. But the fat content of samples markedly lowered the volatility of androstenone as shown in Fig. 1. A rough fractionation on a column of silica followed by steam distillation gave satisfactory results, however, the method was left in favour of the simpler technique of saponification.

(2) *Separation after saponification.* The work up comprises 3 or 4 steps, cf. Fig. 2. Extraction of adipose tissue with ethyl acetate may be omitted, when there it is not necessary to determine the content of fat in tissue. During saponification, only a small part of androstenone is lost. A loss of 35% of $^3H$-activity of the standard added, found at the beginning of these experiments, was caused by $^3H$-exchange. Using a pretreated $^3H$-standard, this effect was eliminated.

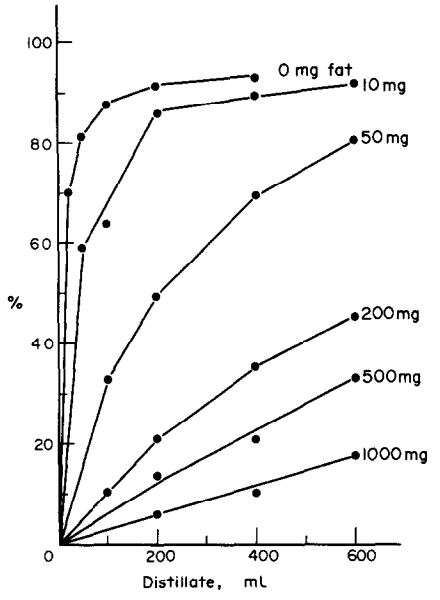


Fig. 1. Separation of 5α-androst-16-en-3-one (androstenone) by steam distillation. 10 μg androstenone-$^3$H were mixed with 10 μg progesterone, testosterone and androst-4-en-3,17-dione each and with the stated amounts of pork fat, distilled off with steam and extracted with chloroform.

Formation of emulsions during extraction was prevented by using 70% aq. methanol to remove polar products of saponification.

When the unsaponifiable fraction was purified by column chromatography on silica gel, the androstenone fraction obtained in good yield contained cholesterol as impurity, so that the g.l.c. column must be reconditioned after some injections. Therefore, instead of column chromatography, t.l.c. was used, resulting in somewhat lower yields but purer androstenone fractions.

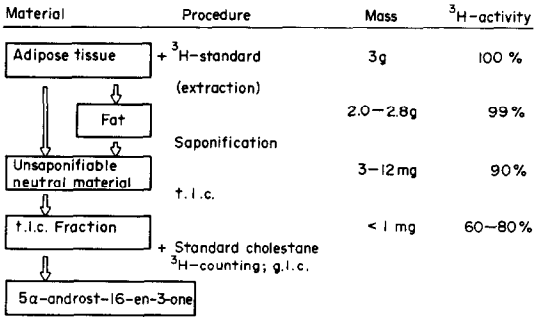


Fig. 2.

The areas of g.l.c. peaks were measured considering a baseline drift and overlapping of neighbouring peaks for samples containing small amounts of androstenone, as shown in Fig. 3 for samples with (a) moderate and (b) small contents of androstenone.

(3) *Accuracy of determination.* The androstenone from boar fat had the same retention times as the synthetic substance, on both QF 1 and XE 60 columns. Two samples isolated after g.l.c. gave I.R. spectra equal to that of synthetic androstenone. After addition of androstenone to the fat from a castrated male, the values listed in Table 1 were obtained. Regression analysis gave the function $y = 0.974x + 0.057$ for recovery of added amounts of $x$ (in μg per 3 g tissue). When only the more precise values of 0.6 to 4.5 μg were used, the function $y = 0.966x + 0.085$ was obtained.

(4) *Reproducibility.* Standard deviations of the triple determinations given in Table 1 correspond to 0.004–0.08 μg androstenone per g tissue. Without the two smallest values, the coefficients of variation were smaller than 10%. Determination of androstenone in adipose tissue of boars resulted in similar reproducibility, as shown in Table 2. However when another

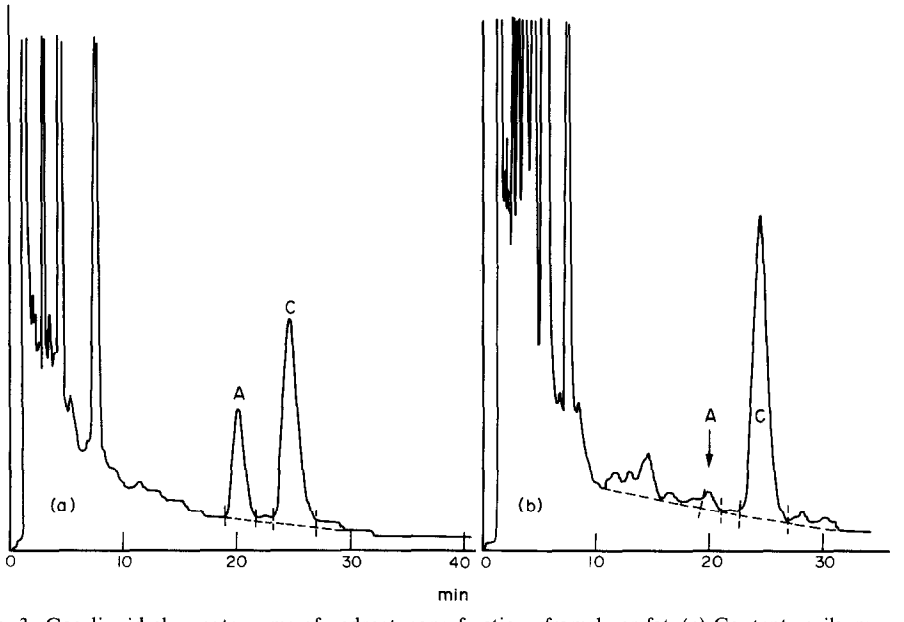


Fig. 3. Gas–liquid chromatograms of androstenone fractions from boar fat. (a) Content easily measurable (0.60 μg/g tissue). (b) Content very low (0.06 μg/g). A = androstenone, C = cholestane.

Table 1. Recovery of 5α-androst-16-en-3-one added to hog fat extract. Values from 3 separate determinations. 2.58 g fat extract. ≙ 3 g adipose tissue

| Added [μg] | Found [μg] Mean ± S.D. | Coefficient of variation [%] |
|---|---|---|
| 0 | 0.057 ± 0.012 | 21 |
| 0.30 | 0.317 ± 0.116 | 37 |
| 0.60 | 0.627 ± 0.021 | 3.4 |
| 1.50 | 1.573 ± 0.119 | 7.6 |
| 3.00 | 3.003 ± 0.225 | 7.5 |
| 4.50 | 4.410 ± 0.056 | 1.3 |

3 g sample was cut off from the same piece of adipose tissue, the deviation in androstenone content was larger, as listed in parentheses for samples 4 to 6. Probably an unhomogeneous distribution of androstenone in adipose tissue is the cause. This error may be reduced by mixing a larger sample.

(5) *Sensitivity.* The method permitted detection of concentrations down to 0.06 and 0.02 μg androstenone per g tissue. But sometimes in this range measuring of androstenone was complicated by overlapping peaks (cf. Fig. 3b), the size of which varied from one animal to another. Also from the values given in Table 1 it can be concluded that concentrations of about 0.03 μg/g tissue can still be detected.

(6) *Volatility as source of errors.* Losses of androstenone during work-up (20–40%) were corrected by measuring the $^3$H-standard. It was only after taking the aliquot for counting $^3$H-activity that an uncorrected loss of androstenone was possible, especially during evaporation of the enriched samples under a current of nitrogen. Therefore, volatility of androstenone under these conditions was studied (Fig. 4). During evaporation of the solvent (about 2 min) no loss was detectable. Losses during drying *in vacuo* were also very small. However the volatility of dry androstenone under the current of nitrogen was considerable. Therefore, to avoid errors, samples for g.l.c. were left under nitrogen only until dryness.

(7) *Content of androstenone in pork fat.* In adipose tissue of 22 boars (age 10–12 months, 80–120 kg live weight) the values of androstenone ranged from 0.08 to 2.45 μg per g tissue (mean ± S.D. 0.85 ± 0.59 μg/g). In 6 castrated males we found 0.02 to 0.06 μg/g (mean 0.03 μg/g), in 2 sows 0.02 and 0.04 μg/g. With reference to fat extract, the values have to be corrected by multiplying by a factor of 1.1 to 1.5.

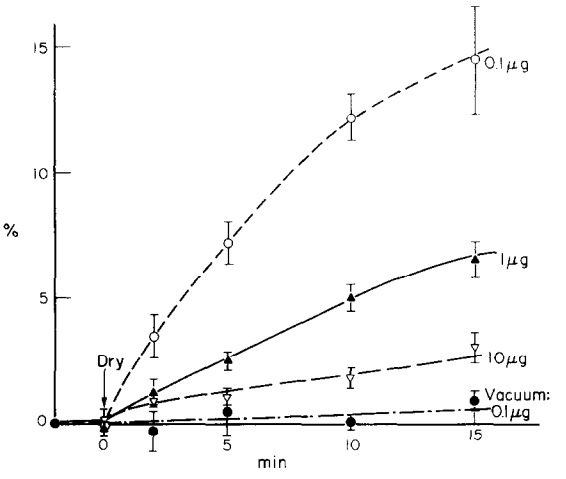


Fig. 4. Losses of androstenone by volatility under a current of nitrogen. Starting solutions of 0.1 to 10 μg androstenone in 0.2 ml hexane–chloroform (9:1 v/v), tubes 6 × 35 mm with conic end, nitrogen 0.1 l/min, temperature 30°C. For comparison: volatility *in vacuo* (25 Torr, 30°C, 10 ml flasks). M ± S.M. from 6 measurements each.

## DISCUSSION

The method described permits measurement of the main sex odour substance in adipose tissue of boars in only three steps. After saponification, partition of the products between hexane and 70% aq. methanol is essential, because it prevents formation of emulsions. The method may be simplified further by omitting the $^3$H-standard and calculating a constant recovery of androstenone (65%). Reproducibility and accuracy of the method appear to be satisfactory. The regression line deviates only slightly from the expected one. This is due to a slight content of androstenone in the tissue used and to volatility. In treating enriched samples, here as with other techniques, the volatility of androst-16-enes under a current of gas and with steam should always be considered.

A precise quantitative determination of androstenone is of practical interest in the screening of meat. In comparison with castrated animals, boars have a

Table 2. Reproducibility of determination of 5α-androst-16-en-3-one in adipose tissue of boars. The values in parantheses were obtained from the first mean and two additional determinations in 3 g samples each collected separately

| Boar No. | 5α-Androst-16-en-3-one [μg/g tissue] Mean ± S.D., n = 3 | | Coefficient of variation [%] | |
|---|---|---|---|---|
| 1 | 1.447 ± 0.042 | | 2.9 | |
| 2 | 1.053 ± 0.040 | | 3.8 | |
| 3 | 0.990 ± 0.026 | | 2.7 | |
| 4 | 0.440 ± 0.028 | (0.637 ± 0.430) | 6.4 | (67.5) |
| 5 | 1.023 ± 0.015 | (1.050 ± 0.079) | 1.5 | ( 7.5) |
| 6 | 1.563 ± 0.090 | (1.470 ± 0.229) | 5.8 | (15.6) |

number of advantages: higher daily weight gain, better feed efficiency, higher percentages of meat and less fat in carcasses [5, 15]. Only the sex odour of boar meat prevents its use for human consumption. Experiments were undertaken to reduce this odour by application of estrogens [16–19], progestagens [18–22] or the antiandrogen cyproterone acetate [22], testing samples by smell and taste. A quantitative determination of androstenone in adipose tissue and also in blood plasma [23] helps evaluate the success with applied substances or breeding manipulations in solving this problem.

The androstenone content in fat of boars was found to range widely between 0.08 and 2.45 μg per g tissue. This in accord to the findings of Claus[5, 6] and Fuchs[4], who found in adult boars 1.03 to 7.49 μg androstenone per g fat extract and 0.3 to 8.0 μg/g, respectively. Recently, Andresen[8] found a mean of 2.09 μg/g adipose tissue, and Claus[24] described values of 0.62 to 0.8 μg/g after maxima of 2.9 to 6.4 μg/g during puberty. In sows and gilts 0.15–0.20 μg/g were found [7], but mostly no androstenone was detectable (<0.09 μg/g) [8]. From our findings of 0.02 to 0.06 μg/g in gilts and sows we conclude that at least 0.1 μg androstenone per g adipose tissue are acceptable when boar meat is provided for human consumption.

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## REFERENCES

1. Gower D. B.: *J. steroid Biochem.* **3** (1972) 45–103.
2. Patterson R. L. S.: *J. Sci. Fd Agric.* **19.** (1968) 31–38.
3. Beery K. E. and Sink J. D.: *J. Endocr.* **51** (1971) 223–224.
4. Fuchs G.: *Swedish J. agric. Res.* **1** (1972) 233–237.
5. Claus R.: Dissertat. Fak. Landwirtsch. Techn. Univ. München 1970.
6. Claus R., Hoffmann B. and Karg H.: *J. anim. Sci.* **33** (1971) 1293–1297.
7. Claus R.: *C.r. hebd. séanc. Acad. Sci. Paris*, Ser. D, **278** (1974) 299–302.
8. Andresen O.: *Acta endocr., Copenh.* **79** (1975) 619–624.
9. Shapiro R. H. and Heath M. J.: *J. Am. chem. Soc.* **89** (1967) 5734–5735.
10. Schönecker B., Ponsold K. and Neuland P.: *Z. Chem.* **10** (1970) 221–222.
11. Radt F. (Ed.): *Elsevier's Encyclopaedia of Organic Chemistry.* Springer-Verlag Berlin-Göttingen-Heidelberg, Series III, Vol. 14 Suppl. (1959), p. 1506.
12. Djerassi C., Engle R. R. and Bowers A.: *J. org. Chem.* **21** (1956) 1547–1549.
13. cf. Ref. 11., p. 2396.
14. Wilzbach K. E.: *J. Am. chem. Soc.* **79** (1957) 1013.
15. Horst P.: *Tierzüchter* **22** (1970) 107.
16. Teague H. S., Plimpton R. F., Cahill V. R., Grifo A. P. and Kunkle L. E.: *J. anim. Sci.* **23** (1964) 332–338; Plimpton Jr., R. F. Ockerman H. W., Teague H. S., Grifo Jr., A. P. and Cahill V. R.: *ibid.* **32** (1973) 51–56.
17. Newell J. A., Tucker L. H., Stinson G. C. and Bowland J. P.: *Can. J. anim. Sci.* **53** (1973) 205–210.
18. Busch W., Sajonski H., Polster H., Ertl G. and Lukas K.: *Fortpfl. Haustiere* **5** (1969) 345–352.
19. Rommel P., Otto E. and Blödow G.: *Monatsh. Veterinärmed.* **29** (1974) 328–332.
20. Jöchle W. and Schilling E.: *J. Reprod. Fert.* **10** (1965) 287–288.
21. Claussen C., Zieger M. and Schimke E.: *Arch. exp. Veterinärmed.* **26** (1972) 981–988.
22. Horst P. and Bader J.: *Züchtungskunde* **41** (1969) 248–261.
23. Andresen O.: *Acta endocr., Copenh.* **76** (1974) 377–387; **78** (1975) 385–391.
24. Claus R.: *Z. Tierzüchtg. Züchtungsbiol.* **92** (1975) 118–126.