Time-Resolved Fluoroimmunoassay for the Measurement of Androstenone in Porcine Serum and Fat Samples

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A simple competitive time-resolved fluoroimmunoassay was developed for the analysis of the boar taint steroid 5α-androst-16-en-3-one in serum and fat samples. Sensitivities of 30 ng/g and 0.8 ng/mL were obtained for fat and serum measurements, and the assays had mean recoveries of 97% and 103%, respectively. The average coefficients for intra- and interassay variation in fat analysis were 6.0%/8.9%, and in serum measurements 5.4%/8.1%, respectively. Boar fat samples contained androstenone in the range of 130−11570 ng/g (n = 106), and in the 108 kg boar group, 58% of samples exhibited fat androstenone levels above 500 ng/g. The serum androstenone concentrations in boars ranged from 1.5 to 237.3 ng/mL (n = 154), but were almost exclusively below the assay range in barrows (n = 124) and gilts (n = 83). A significant ($p < 0.0001$) correlation was observed between boar serum and fat androstenone concentrations (r = 0.89, n = 102). A direct serum analysis seems to give a reliable indication of the androstenone level in fat and could be useful in the routine screening for boar taint.

Keywords: *Androstenone; boar taint; pigs; time-resolved fluoroimmunoassay*

INTRODUCTION

The meat derived from uncastrated male pigs sometimes emits a strong off-odor, referred to as "boar taint", upon heating. Despite the fact that boars are known to have more favorable fattening performance and carcass traits than barrows (castrated males), the majority of male piglets are castrated in order to prevent the occasional presence of taint. The main compounds associated with this problem are androstenone (5α-androst-16-en-3-one) (Patterson, 1968) and microbially derived skatole (3-methylindole) (Vold, 1970; Walstra and Maarse, 1970). Androstenone and other 16-unsaturated C_{19} steroids are synthesized in the testes of boars and released into the blood via the spermatic vein. Due to the lipophilic nature of androstenone, it is taken up by adipose tissue and stored in the fat (Brooks and Pearson, 1986).

If the advantages of boar production are to be exploited, fast and reliable methods are needed for the screening of tainted carcasses as consumers cannot be confronted with unacceptable levels of taint. Immunological assays are well suited for androstenone analysis, because they are simple and rapid to perform and offer the possibility of screening a large number of samples. The analysis is usually performed using fat samples and involves laborious extraction procedures, while blood samples could be used directly without extraction. However, the correlation between the levels of androstenone in blood and fat needs to be determined. A close relationship has already been established with the levels of blood and fat skatole (Claus *et al.*, 1994; Tuomola *et al.*, 1996).

The aim of the current study was to develop a competitive time-resolved fluoroimmunoassay for androstenone based on dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA), and to obtain information regarding the levels of androstenone in the fat and serum samples of Finnish pigs.

MATERIALS AND METHODS

Chemicals and Instrumentation. The steroids were obtained from Sigma Chemical Co. (St. Louis, MO) or Steraloids Inc. (Wilton, NH). ^{1}H NMR spectra were recorded on a Jeol JNM-GX400 FT-NMR spectrometer (Tokyo, Japan) using tetramethylsilane as internal standard. Infrared spectra were determined on a Perkin-Elmer 1600 FTIR spectrometer (Norwalk, CT). HPLC purification of the europium-labeled androstenone derivative was performed using Gradient Pump 2249, UV-monitor Uvicord SII, and Superdex 75 column, all from Pharmacia (Uppsala, Sweden). The purified IgG fraction from rabbit antiserum against androstenone 3-(*O*-carboxymethyl)oxime-BSA conjugate was a gift from Dr. Storm (Intervet International B.V., The Netherlands). Microtitration plates coated with goat anti-rabbit IgG, Delfia Assay Buffer, Delfia Wash Solution, Delfia Enhancement Solution, and Delfia 1234 Fluorometer were from Wallac Oy (Turku, Finland).

Sample Material. Samples were collected at slaughter from 87 young boars, 122 barrows, and 83 gilts, all of which had a weight of about 108 kg. Additional samples were obtained from 69 heavier mature boars. Blood samples (approximately 10 mL) were collected during exsanguination and allowed to clot for 1 h. Serum was then isolated by centrifugation and stored at −20 °C. Boar fat samples were collected from the neck region and stored at −20 °C.

Preparation of Androstenone 3-(*O*-Carboxymethyl)-oxime and Europium-Labeled Androstenone. The synthesis scheme is outlined in Figure 1. Androstenone (**1**) (0.0653 mmol; 17.8 mg) was dissolved in ethanol (3 mL). Carboxymethoxylamine hemihydrochloride (0.069 mmol; 15 mg) and sodium acetate (0.122 mmol; 10 mg) were added to the solution and refluxed for 2 h, after which the solvent was evaporated. The residue was dissolved in water (10 mL) and made alkaline with 10% K_2CO_3 to pH 9. The solution was extracted using diethyl ether (2 × 10 mL) and acidified to pH 1 with 10% HCl, which resulted in a white precipitate of androstenone 3-(*O*-carboxymethyl)oxime (**2**). The product was used without further purification. Spectral data: ^{1}H NMR

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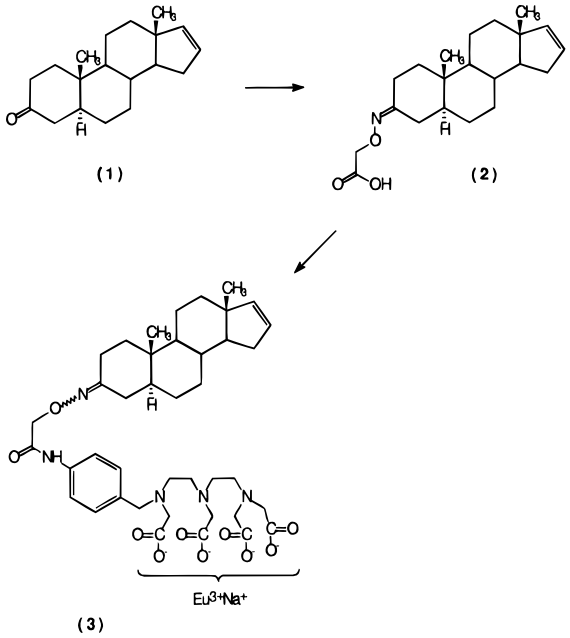

Figure 1. Synthesis scheme for europium-labeled androstenone 3-(*O*-carboxymethyl)oxime.

($CDCl_3$) δ 5.83 (dd, 1 H, 17-olefinic), 5.69 (m, 1 H, 16-olefinic), 4.58 (s, 2 H, CH_2), 3.20 and 2.98 (m, m, 1 H, equatorial 2-H and 4-H, respectively), 0.94 (s, 3 H, 19-CH_3), 0.77 (s, 3 H, 18-CH_3). IR ($CHCl_3$) cm^{-1} 2929, 1733, 1096.

Androstenone 3-(*O*-carboxymethyl)oxime was dissolved in 1,4-dioxane. The europium chelate of 4-aminobenzyldiethylenetriaminetetraacetic acid (Mukkala *et al*., 1989), dissolved in 0.5 M 4-morpholineethanesulfonic acid buffer, pH 5.5, was added to the carboxysteroid solution. 1-(3-(Dimethylamino)-propyl)ethylcarbodiimide hydrochloride was added to the reaction mixture and stirred for 30 min (Mikola and Miettinen, 1991). The product was purified by HPLC (eluent 20% CH_3-CN, 0.05 M NaCl, 0.05 M Tris-HCl; flow rate 0.8 mL/min).

Sample Preparation. Fat samples were melted in a microwave oven or thermal block (60 °C) and 30 μL of liquid fat was transferred to a capped tube containing 500 μL of methanol. The sample was extracted for 30 min (55 °C) and mixed once during the incubation. The sample was allowed to cool for 10 min at room temperature and a 50 μL aliquot of the methanol extract was diluted 1:9 with assay buffer and analyzed. Serum samples were diluted 1:6 with buffer and analyzed directly without extraction or purification. Assay standards were prepared by spiking gilt or barrow samples with known amounts of androstenone (serum, 2, 5, 20, 50, and 100 ng/mL; fat, 50, 200, 500, 2000, and 5000 ng/g). Samples having concentrations beyond the working range were reassayed after dilution with the zero calibrator.

Isolation of Unconjugated Serum Steroids. Conjugated and unconjugated serum steroids from 31 boar samples were isolated according to the method of Payne *et al.* (1989). Briefly, serum samples and standards were denatured with urea at 60 °C and applied to Sep-Pak C_{18} cartridges (Waters, Milford, MA). Conjugated and unconjugated fractions were collected by sequential elution with 47% and 100% methanol, respectively. The fractions were evaporated to dryness and reconstituted in assay buffer (pH 7.75).

Time-Resolved Fluoroimmunoassay. All samples were analyzed in duplicate. Fifty microliters of sample and 50 μL of europium-labeled androstenone in suitable dilution were pipetted into anti-rabbit coated (secondary antibody) microtitration wells. Diluted primary antibody, 50 μL,was added, and after incubation (1.5 h) at room temperature, the wells were washed six times with wash solution (pH 7.75). Enhancement solution (200 μL) was added, and the wells were shaken for 5 min at room temperature. The enhanced fluorescence was measured in a time-resolved fluorometer.

Evaluation of Assay Performance. The specificity of the assay was assessed by measuring the cross-reactivity of the related steroids. Cross-reactivity was expressed as the percentage ratio of the amounts of androstenone and the tested substance needed to cause 50% displacement of tracer. The assay sensitivity was defined as the concentration corresponding to the mean signal of replicates of the zero standard (serum, $n = 24$; fat, $n = 12$) plus three times the standard deviation. The serum assay accuracy was determined by spiking a gilt sample (androstenone, 0 ng/mL) with doses of 5 and 50 ng/mL and boar serum pool (25.1 ng/mL) with 20 and 50 ng/mL of androstenone, and calculating the recovery ($n = 6$). The fat assay accuracy was determined in the same manner with samples containing 0, 220, or 1120 ng of androstenone/g spiked with doses of 250 or 500 ng/g ($n = 3$). Method linearity was assessed by analyzing serial dilutions of five serum and fat samples containing 75.7−91.6 ng/mL or 3200−5100 ng/g of androstenone, respectively. The intraassay repeatability and interassay reproducibility were studied with repeated analyses ($n = 10$) of three samples. The time-related bias (drift) was controlled by including calibrator samples within each sample batch.

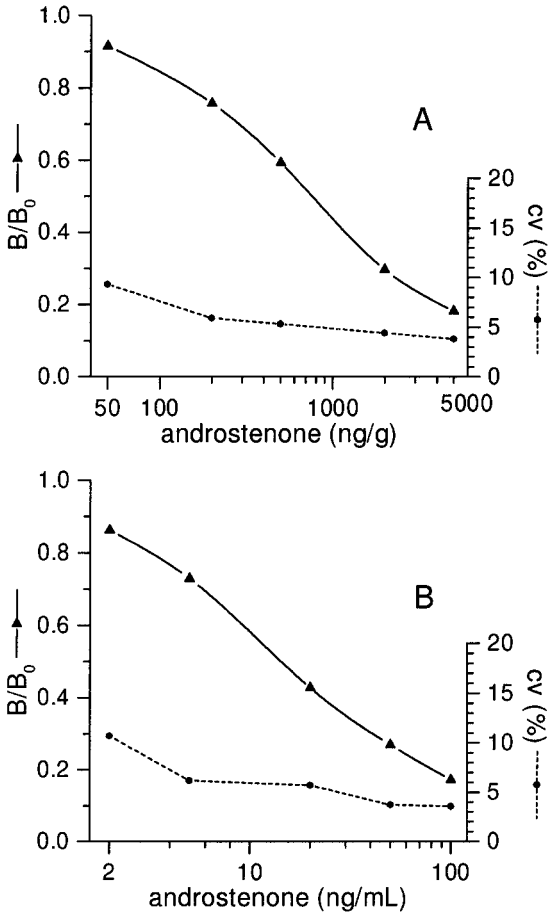

Figure 2. Standard curve and precision profile of fat (A) and serum (B) androstenone assay ($n = 10$).

Statistical Analysis. The data were analyzed using STATISTICA for Windows, version 5.1 (StatSoft Inc., Tulsa, OK). The skewed distributions of androstenone were normalized by logarithmic transformation, and transformed data were used for correlation studies. Significant differences among nontransformed data were identified using nonparametric Mann-Whitney U-test.

RESULTS

Assay Characterization. The standard curves and precision profiles of the fat and serum androstenone assays are presented in Figure 2 (10 replicates). The fat androstenone assay had a working range of 50−3000 ng/g with a sensitivity of 30 ng/g. Serum androstenone was measured in the range of 1.0−90.0 ng/mL with a

Table 1. Precision of the Serum and Fat Androstenone Assays

concn	cv % intra-assay	cv % interassay cv%
serum, ng/mL	(n = 10)	(n = 10)
16.3	7.6	6.3
30.5	3.6	7.2
124.5	5.0	10.8
fat, ng/g	(n = 12)	(n = 10)
256	6.3	12.6
504	6.7	7.5
1324	5.1	6.7

Table 2. Specificity of the Antiserum

compound	cross-reactivity (%)
5α-androst-16-en-3-one	100.0
5α-androst-16-en-3β-ol	10.4
5α-androst-16-en-3α-ol	6.3
4-androstene-3,17-dione	3.4
5α-dihydrotestosterone	0.5
testosterone	0.4
17β-estradiol	<0.02

Table 3. Androstenone Concentrations in Boar Fat and Serum Samples

group	young boars	old boars
fat samples		
number of samples	69	37
range (ng/g)	130−3780	550−11570
median (ng/g)	670	3650
serum samples		
number of samples	85	69
range (ng/mL)	1.5−126.3	15.4−237.3
median (ng/mL)	16.4	76.9

sensitivity of 0.8 ng/mL. The quantitation limit of 1.0 ng/mL was validated with fortification experiments, and the recovery of the spiked serum samples was 109.7% with a variation coefficient of 17.5% (n = 8). In the serum assay accuracy studies, 95.2−112.3% (average 103.0%) of added androstenone was recovered. The assay showed no appreciable matrix effects as the regression line of the dilution series was linear (r = 0.998, p < 0.001) with a slope of 0.985 (95% confidence interval 0.961−1.010). The fat androstenone measurement had an average recovery of 97.0% (range 84.4−107.2%). The regression line of the fat sample dilution series was also linear (r = 0.994, p < 0.001) with a slope of 0.981 (95% confidence interval 0.953−1.008). Assay precision is given in Table 1. The average coefficients for intra- and interassay variation in serum measurements were 5.4% and 8.1%, respectively. The respective coefficients in fat analysis were 6.0% and 8.9%. The cross-reactivity of the antiserum is summarized in Table 2. 5α-Androst-16-en-3β-ol, 5α-androst-16-en-3α-ol, and 4-androstene-3,17-dione showed cross-reactivity, but the recognition of other tested steroids was not significant.

Application to Porcine Serum and Fat Samples. The androstenone concentrations in boar fat and serum samples are presented in Table 3. The percentages of young boars which exhibited fat androstenone levels above 500 or 1000 ng/g, were 58% and 13%, respectively. Both fat and serum levels were significantly (p < 0.0001) higher in the old boar group than in the young boar group. Concentrations in the barrow (n = 124) and gilt (n = 83) serum samples were almost exclusively below the assay range, with one exception in both groups (2.2 and 1.5 ng/mL, respectively). A significant (p < 0.0001) linear correlation was observed between serum and fat androstenone concentrations in the young boar group (r = 0.86, n = 65), old boar group (r = 0.73, n = 37), and all boars (r = 0.89, n = 102) (Figure 3). The results obtained by direct serum analysis were closely related with the total androstenone levels of unconjugated steroid fraction (r = 0.98, p < 0.0001) (Figure 4). Regression analysis of the results gave the equation $y = 1.088x - 3.366$. The applicability of the solid phase extraction method for androstenone was verified with the analysis of serum standards. The results confirmed the androstenone to elute in the unconjugated steroid fraction only.

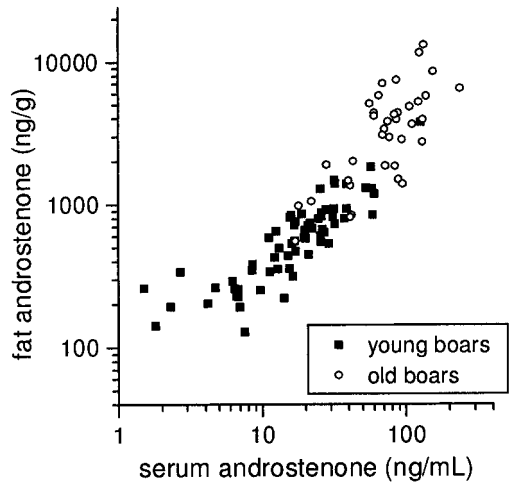

Figure 3. Relationship between fat and serum androstenone concentrations (n = 102).

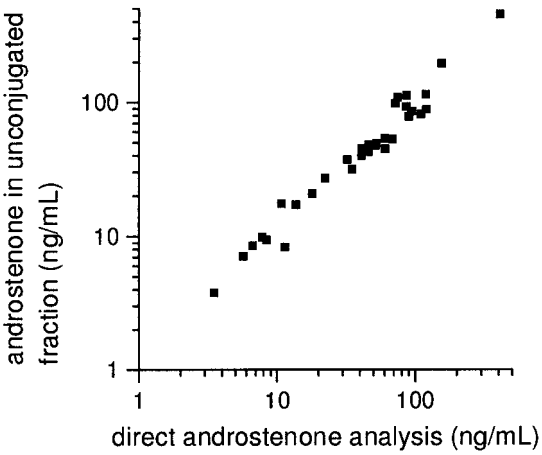

Figure 4. Relationship between directly measured serum androstenone and androstenone level in the extracted unconjugated steroid fraction.

DISCUSSION

Assay Characteristics. The first immunological methods for androstenone analysis were based on radioimmunoassay (RIA) (Claus, 1974; Andresen, 1974, 1975), and they involved the disadvantages connected with potentially hazardous radioactive materials. RIAs were later on largely replaced by non-radioisotopic alternatives, especially enzyme-linked immunosorbent assay (ELISA) (Claus *et al.*, 1988; Asghar *et al.*, 1988; Abouzied *et al.*, 1990; Boutten *et al.*, 1994). Enzymes used in the ELISA systems have the general advantage of a high signal amplification, but the signal is very dependent on incubation time, temperature, and other physical and chemical conditions during the substrate incubation. They are also sensitive to interfering substances in samples, such as endogenic enzymes or

inhibitors. Fluorescent labels offer an alternative in the field of non-radioisotopic detection but the sensitivity of the assay is limited by background interference. This interference can be avoided using time-resolved fluorometry (for review, see Hemmilä, 1991). Some lanthanide ions, such as Eu^{3+}, form highly fluorescent chelates with certain organic ligands. The lanthanide fluorescence has an exceptionally large Stokes' shift (the difference between the excitation and emission wavelengths), narrow emission band, long decay time, and high quantum yield. In the Delfia system, a nonfluorescent chelate is employed to bind europium to the analyte (Hemmilä, 1991). After the bioaffinity reaction is completed, the europium ions are dissociated from the chelates by means of an enhancement solution in which the lanthanide ions form new fluorescent complexes with β-diketone. Time-resolved fluorometry involves measurement of light at a fixed time after the fluorophore has been excited. By this time, background fluorescence has died away.

The *O*-carboxymethyl oxime functionality is widely used in steroid immunoassays, and it was used here in the synthesis of europium-labeled androstenone (Figure 1). The oxo group of the steroid reacts readily with the aminooxy group of carboxymethoxylamine in alkaline ethanol solution to produce a stable oxime bond. Due to the double bond C=N formed, two isomers differing in the *E*/*Z* configuration were obtained. According to the ^{1}H NMR data the ratio between *E* and *Z* isomers was about 0.9. The structure of androstenone 3-(*O*-carboxymethyl)oxime was proven by ^{1}H NMR and IR spectra. In ^{1}H NMR the characteristic signal of OCH_2-COO was at 4.58 and the signals of equatorial C-2 and C-4 hydrogens of *Z* and *E* isomers (Oka and Hara, 1968) were at 3.20 and 2.98, respectively. In IR spectra the C=O absorption of the oxo group at 1715 cm^{-1} was shifted to 1733 cm^{-1} of the carboxyl C=O. The isomers were not separated, and the mixture was labeled with a europium chelate as described previously (Mikola and Miettinen, 1991). Instead of TLC separation, we used HPLC with a Superdex 75 column to purify the labeled androstenone 3-(*O*-carboxymethyl)oxime (details of the purification will be reported elsewhere).

All published methods for the production of androstenone antibodies share the common strategy of using androstenone 3-(*O*-carboxymethyl)oxime derivatives as immunogens. Thus, cross-reactivities in all studies are quite similar (Claus, 1974; Claus and Giménez, 1977; Hobe *et al*., 1982; Claus *et al*., 1988), although more specific antiserum (Andresen, 1974) and less specific monoclonal antibodies (Abouzied *et al*., 1990) have also been reported. Consistent with the fact that antibodies usually best recognize structures distal to the site of hapten attachment to the carrier, the double bond between carbons 16 and 17 was found to be important for recognition, and any modifications in the D-ring structure largely reduced antibody binding. On the other hand, structures close to the carrier are often not very antigenic, but some recognition took place as the antibodies were able to discriminate between the α- and β-orientation of the hydroxyl group at the C3-position. The specificity could still be increased by coupling the steroids to carrier via positions on the steroid which are not subject to metabolic change and are remote from distinguishing functional groups. Carbons 6 and 7 in the B-ring have been suggested for this purpose (Midgley and Niswender, 1970), and good results have been obtained with, for example, dihydrotestosterone (Condom and Desfosses, 1977). From the tested cross-reactive compounds, only 5α-androst-16-en-3α-ol (An-α) may be of practical importance. The concentrations of 16-unsaturated C_{19}-steroids other than androstenone have not been extensively studied in the peripheral plasma, but An-α concentrations equal to the levels reported usually for androstenone have been reported (Bicknell and Gower, 1976). In a study involving three boars, only a low concentration of 5α-androst-16-en-3β-ol (An-β) was observed in plasma, but An-α was found in even higher amounts than androstenone (Claus and Hoffmann, 1971) and may thus contribute to the estimated levels of steroid in boar blood. This cross-reaction does not play any significant role in fat samples where androstenone concentration clearly exceed the levels of androstenols (Claus, 1979; Brennan *et al*., 1986; García-Regueiro and Diaz, 1989). This is further confirmed by the high correlation observed between the results of radioimmunological analysis and gas chromatography combined with supercritical extraction and mass spectrometry (Mågård *et al*., 1995).

An extraction step is required in the fat sample preparation. Strong organic solvents have been used for this purpose (Andresen, 1975; Claus *et al*., 1988; Meloen *et al*., 1994), but extra steps such as solvent-solvent extraction and evaporation are required leading to increased between-sample variation and slow sample turnaround time. The use of water-miscible solvents, especially methanol (Boutten *et al*., 1994) and ethanol (Asghar *et al*., 1988) eliminate the need for these steps as extracts can be directly assayed after dilution with buffer. In preliminary experiments, ethanol and 2-propanol were tested for this purpose but were found to have a major effect on antibody binding. In contrast, a high amount of methanol could be introduced in the bioaffinity reaction without compromising the assay performance, which is in accordance with the results of Boutten *et al*. (1994). The method uses only 0.5 mL of organic solvent per sample compared with the volumes of 3−10 mL needed in the previously published immunoassays (Claus, 1974; Andresen, 1975; Claus *et al*., 1988).

The procedural losses were compensated for by preparing the calibration curve using fat from a female pig. To ensure the minimal level of endogenous androstenone in the zero-calibrator it was chosen based on to its ability to give a maximal detection response in the assay. Despite this, it should be noted that a small endogenous amount of androstenone is probably present (<100 ng/g) thus leading to a small systematical bias (Claus *et al*., 1988). The same also applies to serum assay standardization, where the usage of barrow serum as a zero calibrator resulted in a systematical underestimation of serum androstenone in the order of 1−2 ng/mL (Andresen, 1974, 1975; Hobe *et al*., 1982). The serum samples were assayed directly after dilution, and as shown by the accuracy studies, no matrix effects were observed. In the radioimmunoassay of Booth *et al*. (1986) boar plasma samples were analyzed directly without any dilution (recoveries not reported). On the other hand, the enzyme immunoassay of Abouzied *et al*. (1990) was found to have a marked matrix interference from boar serum samples, and a 100-fold dilution of samples was needed to obtain sufficient recovery. The TR-FIA procedure used in our study showed good precision regardless of sample type. One factor contributing to this was the use of secondary anti-rabbit antibodies in the coating of the wells. This approach

avoided the direct coating of any component critical in the competitive reaction and resulted in a small variation in the amount of immobilized and immunoreactive anti-androstenone antibody. The variation in the amount of immunoreactive components is more difficult to control when the primary antibodies or antigen-carrier conjugates are directly immobilized on to the microtitration well surfaces.

Androstenone Levels in Pigs. The fat androstenone concentrations found in boars were within the range usually reported (*e.g.*, Malmfors and Andresen, 1975; Patterson and Lightfoot, 1984; Brennan *et al.*, 1986; Claus *et al.*, 1988; Mågård *et al.*, 1995). Fat androstenone levels are highly dependent on genetic factors that affect both sexual maturity and potential for androstenone production. In most young boars, a marked increase in androstenone production is observed around puberty (Claus, 1975; Andresen, 1976), and the high individual variability of fat androstenone concentrations can be partly attributed to the differences in the onset of adolescence (Bonneau, 1982; Claus *et al.*, 1994). Due to the high individual variation, the correlation between weight and androstenone level has usually not been significant, but consistent with the current study, higher concentrations have been observed with higher liveweights (Andresen, 1976; Bonneau and Desmoulin, 1980; Patterson and Lightfoot, 1984; Brennan *et al.*, 1986; Bonneau, 1987). Some of the older boars may also have been used for breeding purposes, which has been reported to affect steroid levels (Andresen, 1976; Claus, 1979). The level of fat androstenone resulting in an off-odor unacceptable to consumers has not been firmly established, mainly because human perception of androstenone is under genetic control (Wysocki and Beauchamp, 1984) and varies significantly among consumers. Concentrations of 1000 and 500 ng/g have been used as cutoff levels in the past studies (Booth *et al.*, 1986; Babol and Squires, 1995). From an economical point of view, the need for establishing a reliable sorting limit is emphasized by the fact that a high proportion of young boars had androstenone concentrations in the 500−1000 ng/g range.

The results from the direct serum androstenone analysis were higher than previously reported values from blood samples extracted with organic solvents (0−76 ng/mL) (Bonneau, 1982; Patterson and Lightfoot, 1984). The cross-reactivity studies together with zero levels obtained from over two hundred barrow and gilt samples demonstrate the assay to be very specific, but the possibility of cross-reacting androstenone metabolites still has to be considered. The catabolism of androstenone is known to lead to compounds that are found in both unconjugated and conjugated steroid fractions, but an appreciable proportion of them has still not been identified (Saat *et al.*, 1974; Bonneau and Terqui, 1983). These metabolites are present in the direct analysis but may be poorly recovered to the extracted samples. The selection of extracting solvent always provides limited selectivity to the steroid analysis, as for example, hexane extraction of plasma recovers 85% of progesterone, but only 1% of cortisol and corticosterone (Robards and Towers, 1990). The hypothesis is also consistent with the fact that studies using hexane for extraction (Groth and Claus, 1977; Claus and Hoffmann, 1980; Bonneau *et al.*, 1982; Claus *et al.*, 1983) have constantly led to lower blood androstenone levels than studies using the more polar ethyl acetate (Andresen, 1974, 1975; Malmfors and Andresen, 1975; Patterson and Lightfoot, 1984; Booth *et al.*, 1986). In this study, the total unconjugated steroid fraction was isolated from serum samples using a solid-phase extraction method, which enabled the recovery of steroids having a diverse range of polarities. The results matched closely with those of direct analysis, which is also consistent with the fact that the keto group at the 3-position is not directly available for conjugation.

The correlation between the levels of androstenone in serum and fat has been investigated in several studies and the results have ranged from the lack of correlation (Malmfors and Andresen, 1975; Malmfors *et al.*, 1976; Lundström *et al.*, 1978; Bonneau *et al.*, 1982) to a high degree of association (Andresen, 1976; Groth and Claus, 1977; Hobe *et al.*, 1982; Booth *et al.*, 1986). The close relationship observed in the current study suggests that direct serum measurement can give an indication of the androstenone level in fat. The use of blood samples and direct assays gives distinct advantages over the use of fat samples due to the simplicity and ease of sample preparation and facilitates rapid and sensitive assays with large throughput of samples. To exploit this possibility, the threshold value in blood needs to be established and more research is needed to characterize the relationship between the androstenone concentration in the sera and the intensity of boar taint.

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