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Comparison of two analytical methods of androstenone determination in backfat of pigs, treated with anabolic steroids.

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INTRODUCTION

Fattening of entire male pigs (boars) instead of castrated animals (barrows) presents large economical advantages (1)(2). However, the commercialisation of boar carcasses is hampered by a strong and unpleasant cooking odour, exhibited by some (5-20 % for a 100 kg live weight) of the boar meats. The so-called boar taint has been the subject of numerous investigations. In 1960 Patterson (3) demonstrated that a chemical substance: the steroid hormone androstenone should be responsible for the off-flavour. Since then, several correlations (r= 0.4-0.76) between the androstenone concentration of the fatty tissue and boar taint were obtained (4,5,6,7). Later on, other investigators demonstrated that also other malodorous compounds as indole (8), skatole (9) and volatile aldehydes (10) may have an important contribution to boar taint.

The goal of boar taint research is the commercialisation of boars without taint risk for the consumer. This could be achieved by reduction or elimination of the component(s) responsible for the taint (e.g. androstenone) during fattening or by selection of boar carcasses in the slaughterhouse and directing the tainted ones to other destinations. During fattening, the boar taint and the androstenone level in backfat may be reduced by anabolic treatment. Implantation of boars with diethylstilboestrol (DES) has been proved to reduce sex odor while maintaining the advantagous carcass characteristics(11). However, stilbenes are potential carcinogens and there is a ban on their use as growth promotors in animal fattening.

The determination of androstenone content, as a principal component of boar taint, may be used for selection of tainted carcasses in the slaughterhouse. Immunological methods are most fit for these routine measurements. Radio-immunological methods (e.g. RIA, Andresen (12)(13); Claus (14)) require the use of radionuclides which restricts their use in the slaughterhouse. The non-isotopic immunological detection of androstenone in porc fat would permit simple and low cost screening of tainted carcasses. An ELISA (Enzyme linked Immuno Sorbent Assay) as developped by Storm (15) appeared in some laboratories in an experimental state but is not yet commercially available. However, immunological methods may be sensitive to cross-reactions, leading to less accurate results and are often checked with independent methods.

In this investigation back-fat samples of pigs treated with anabolic steroids in order to test their growth performances and carcass characteristics (16) (Laboratory of Animal Nutrition, RUG) were analysed with two independent methods. The ELISA analysis was performed by the Intervet Laboratory, (Boxmeer). The CGC analysis was carried out in the Laboratory of Chemical Analysis of Food from Animal Origin, RUG. The results of the two methods are compared with each other. The influence of parenteral use of IMPLIX BF ® and of REVALOR ® on the androstenone concentration of back-fat was investigated. On a few samples the influence of hormonal treatment on the positional distribution of the fatty acids within the triglyceride molecule was studied.

MATERIALS AND METHODS

-Animal material

There were two series of experiments

In the first series there were 16 barrows, 16 boars and 16 gilts, each divided into 8 control and 8 implanted animals. The treated barrows were implanted at about 60 kg with 20 mg 17 β oestradiol + 200 mg testosterone (IMPLIX BF ®). The treated boars and gilts were implanted with 20 mg 17 βoestradiol + 140 mg trenbolone acetate (REVALOR ®). The pigs were slaughtered at about 98 kg (160-170 days old) after 40-45 days of implantation. They were individually penned from 10 weeks of age till slaughter. In the second series, 32 boars and 16 gilts were used. Half of the animals of each sex were implanted with REVALOR® at 50-55 kg. In addition another group of 16 gilts were used, half of which were implanted with IMPLIX BF ®. The animals were slaughtered at the same age and weight as in the first series but after a (little bit) longer implantation period (60-65 days). These animals were housed in groups of 4 animals of the same sex and the same treatment.

Immediately after slaughter, samples of the outer layer of the backfat at the 10th rib were taken for androstenone determination. Samples were divided into 2 subsamples and stored at -18°C. The fat samples for androstenone determination with the ELISA method were stored for 9 months before analysis, the samples for the gaschromatographic method were stored for 30 months.

-ELISA method

Androstenone was extracted from fat with help of an automated extraction device with a capacity of extracting and handling 160 fat samples in about 1 hour. Briefly the extraction procedure consisted of the following steps: a piece of fat is melted at 85°C. 0.2 ml of liquid fat is transferred to 0.8 ml light petroleum in a glass tube and mixed. 2 ml methanol-dist water (9:1) is added and the content is mixed again. After separation of the solvent layers 1 ml of the methanol layer is transferred to a clean glass tube. 0.5 ml light petroleum is added and the

content is mixed. After separation of the layers 0.4 ml of the methanol layer is transferred to a clean tube. The methanol is evaporated to dryness at 60°C under nitrogen. The remainder is redissolved in 0.8 ml phosphate buffer.

Androstenone microelisa is an enzyme-immunoassay based on a competition between non labelled and enzyme-labelled androstenone. The wells of polystyrene microelisa plates have been coated with rabbit anti-androstenone immunoglobulins (antibodies to androstenone), which constitutes the solid-phase antibody. The test samples,togheter with a constant amount of enzyme-labelled androstenone are incubated in such a well. Androstenone,if present in the samples will partly inhibit the binding of enzyme-labelled androstenone to the solid-phase antibody. Unbound material is removed by washing. Subsequent addition of the appropriate substrate and chromogen results in development of a blue colour in the test wells,which will shift to a yellow colour after terminating the reaction by addition of sulphuric acid.

If the sample or samples contain androstenone then only part of the enzyme labelled androstenone can be bound and consequently less colour will be produced compared to the zero reference wells.

-Gaschromatographic method

A fat sample (0.4 g) was heated at 80 °C during 15 minutes with 1.4 ml toluene, 2 ml KOH solution (10% in methanol) and 20 µl internal standard solution (AAON ,androstanone (5-α-androstan-3-one). After cooling,the mixture was extracted with 2.5 ml methanol, 2 ml distilled water and 5 ml light petroleum. The upper phase was transferred to conical 5 ml reaction tubes. The solvent was evaporated to dryness under a jet of nitrogen, 5 µl FLOROX reagent ((o-pentafluorobenzyl) hydroxylamine.HCl.2.5 mg/ml in E.C. grade pyridine), Pierce (Rockford,IL.,USA) was added and the tube was heated at 100°C for 1.5 h . After cooling 40 µl n-heptane was added and the tube was heated again for 5 min. at 100°C. 1 µl of the contents was injected into the gas chromatograph.

The gas chromatograph used was a Varian 3700, equipped with a capillary injection system and an ECD. For this series of measurements a 15 meter capillary CP Sil 5 CB (an OV-1,SE-30 analogue, Chrompack,the Netherlands) of 0.1 mm I.D. was used. Hydrogen was used as carrier gas at a rate of 1 ml/min. The splitting ratio was 1 : 60. Nitrogen was used as make-up gas for the ECD at 30 ml/min. The column, injector and detector temperatures were 245 °C, 280 °C and 320 °C respectively. The retention times of the syn and anti forms of the PFBHA derivatives of the steroids were : AEON, 5.7 and 6.1 min; AAON, 6.4 and 6.7 min.

-Statistical calculations and plots

All statistical calculations and plots were performed on a Apple Macintosh Plus computer (Apple computer inc, Cupertino, U.S.A.).The statistical program Statworks (Heyden & Son Ltd,Londen) and the business graphics program MS Chart (Microsoft Corporation, Bellevue, U.S.A) was used.

RESULTS AND DISCUSSION

-Gaschromatographic method

In comparison with earlier investigations (17)(18) the following alterations were made: the saponification time of the fat sample was reduced from one hour to 15 minutes. Instead of cyclohexane, n-heptane was used for taking op the derivatised steroids. The time of the chromatographic run was shortened with ca 3 minutes through use of a narrow-bore capillary of 0.1 mm I.D.

- Growth and carcass composition

The effect of parenteral use of estradiol, progesteron, testosteron and trenbolone on growth and carcass composition was described by R.O.De Wilde and H.Lauwers (16). It could be stated that, in ad libitum feeding conditions, feed intake was reduced by treatment without affecting the growth rate. The carcasses were leaner in the treated animals. Results were more pronounced in barrows than in boars and gilts.

Table I: Descriptive statistics of the boar samples (n = 46) on androstenone content of the backfat.

method/ parameter	ELISA (ppm)	CGC (ppm)
minimum	0,0	0,0
maximum	2,16	1,40
median	0,235	0,150
mean	0,346	0,254
standard deviation	0,438	0,320
skewness	2,35	1,77
kurtosis	6,5	3,0

- Comparison of the two analytical methods.

The two analytical methods were compared with each other on basis of the results obtained with each other on basis of the results obtained with the boar fats only. For gilts and barrows the concentration of androstenone found was always small for both methods. So,these data could not be used to compare the methods. The numerical summary of the results is given in tabel I.

Both populations are not significantly different from a normal distribution (Kolmogorov-Smirnov normality test; CGC $p = 0,07$; ELISA $p = 0,07$) and are not differently distributed ($p = 0,2$). However,with the CGC method significant lower androstenone values are found than with the ELISA method (non-parametric Wilcoxon signed rank test $p \leq 0,001$). This might be explained by differences in accuracy between the two methods or by differences in the length of the storage period of the samples. Since the samples for the gaschromatographic method were stored over a longer period (30 months versus 9 months for the ELISA method) the androstenone concentration may have decreased in function of storage time. The relation between the results obtained with the ELISA method (as dependent variable) and the CGC method (as independent variable) is given in Fig.1.

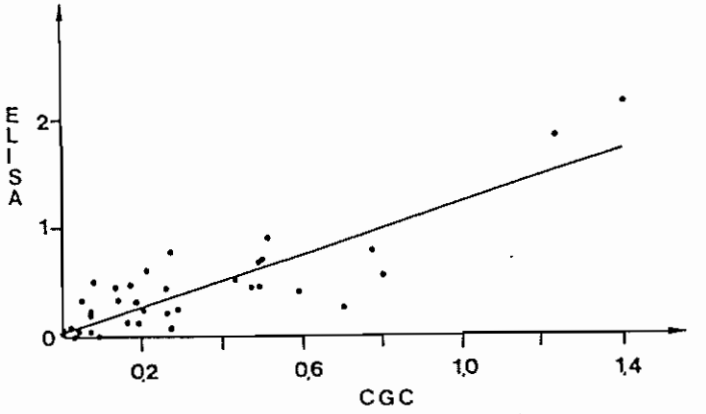

FIG 1 : Regression between the androstenone values determined by the ELISA method (y-axis) and CGC method (x-axis) ($y = 1,20\ x + 0,04$).

Both methods are well correlated. The product-moment correlation coefficient was calculated as $r = 0,881$ ($n = 46$ $p \leq 0,001$). The data used are composed from samples from both control and treated boars. The calculations were also carried out on the control boars only. From the data of the control animals analogeous results were obtained ($r = 0,881$; $n = 22$; $y = 1,2\ x + 0,09$)

-Influence of parenteral use of REVALOR® on androstenone concentration of boars.

The androstenone concentrations in backfat samples of boars treated with REVALOR® were compared with those of control animals. The results are given in tabel II.

Table II: Comparison of the descriptive statistics of the boars, treated with anabolic steroids with control samples ($n = 46$) on androstenone concentration of the backfat

Method	concentration found with ELISA (ppm)		CGC (ppm)	
Parameter	Control	REVALOR®	Control	REVALOR®
minimum	0,19	0,0	0,07	0,0
maximum	2,16	0,5	1,40	0,7
median	0,47	0,05	0,35	0,03
mean	0,63	0,09	0,44	0,09
standard deviation	0,49	0,13	0,35	0,16
skewness	1,97	1,81	1,28	2,64
kurtosis	3,28	2,6	0,93	7,28

The results show clearly that parenteral treatment of boars with REVALOR® significantly reduces the androstenone concentration of the backfat of boars from a mean value of ca 0.5±0.35 ppm to a mean value of ca 0.1 ±0.16 ppm (Student's t test ($p \leq 0,001$)) for both the ELISA and the CGC method).In Fig.2 the analytical results for the two series of experiments are given in scatter diagrams. These figures show that no differences may be found between animals housed in groups of 4 and boars that were individually penned.

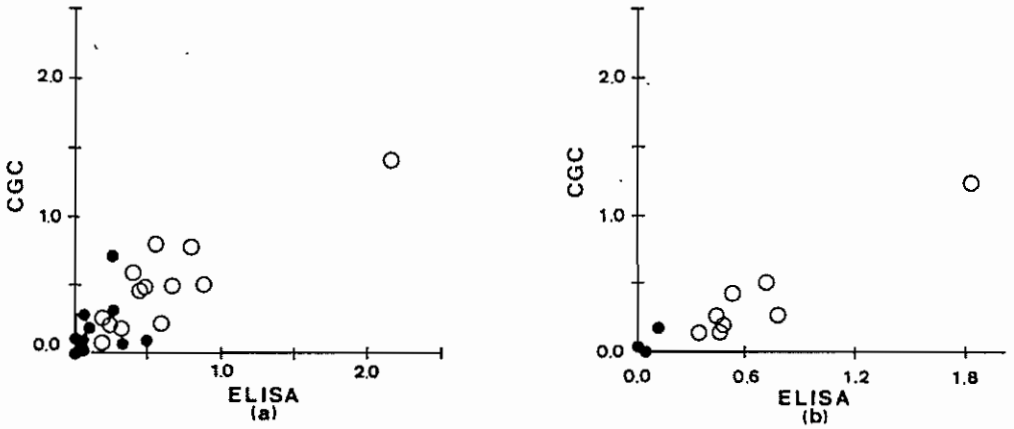

FIG 2 : Scatter diagrams of androstenone values of control boars (○) and boars treated with REVALOR® (●); (a) boars housed in groups of 4 (b) boars individually penned

-Influence of parenteral treatment of barrows with IMPLIX BF ® and gilts with REVALOR® and IMPLIX BF ®

In the 16 barrow fats and the 47 gilts fats analysed small or undetectable concentrations of androstenone were found with both the ELISA and the CGC method. The results of the ELISA analysis are summarized in table III. With the CGC method analogeous results were found. It is obvious that the small androstenone level of barrow fat is not influenced by treatment with IMPLIX BF ®. In the backfat of gilts no change of the androstenone level was found by parenteral treatment with REVALOR® or IMPLIX BF ®

Table III: Comparison of the descriptive statistics of gilts and barrows, treated with anabolic steroids with control samples. (Results of ELISA analysis)

Method	concentration of androstenone in backfat of GILTS (ppm)			BARROWS (ppm)	
Parameter	Control	REVALOR®	IMPLIX	Control	IMPLIX
n	23	16	8	8	8
minimum	0,0	0,0	0,0	0,0	0,0
maximum	0.18	0,11	0,12	0.0	0,2
median	0,0	0,03	0,07	0,0	0,0
mean	0,05	0,03	0,05	0,0	0,04
standard deviation	0,06	0,04	0,05	0,0	0,07

- Influence of hormonal treatment on the positional distribution of fatty acids within the triglycerides.

No data were found on the influence of hormonal treatment on the positional distribution of fatty acids within the triglycerides of pork fat. Therefore the triglyceride (T) and monoglyceride (M) composition of 6 gilt fats (2 control animals, 3 animals treated with REVALOR® and 1 animal treated with IMPLIX®) was analysed with a TLC-CGC method described before (19) (20). From the two fatty acid compositions T and M the identification parameters 3T-2M of the fatty acid C16:0 (palmitic acid) and the proportion in 2-position (PROP-2 =(MG/TG)*33.33) of the fatty acid C18:1 (oleic acid) were calculated. The results are given in Fig 3. This figure shows clearly that the backfat samples taken from gilts treated with REVALOR® or IMPLIX® fall within the cluster formed by our standard pork fats and the two control fats . The parameters used in Fig 3 are species specific for pork fat. Since the parameters of the animals treated with anabolic steroids do not differ from the control ones and our standard pork fats it is very unlikely that hormonal treatment with REVALOR® or IMPLIX® is of any influence on the positional distribution of fatty acids within the triglycerides of pork fat.

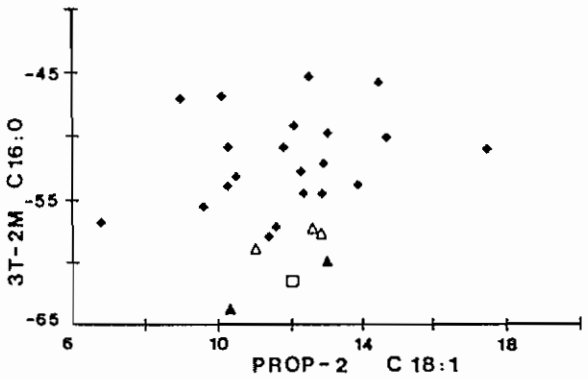

FIG 3 : Comparison of parameters of gilts treated with anabolic steroids with species specific parameters of pork fat ;(◆): standard pork fats ; (▲):control gilts ; (△): gilts treated with REVALOR®;(□): gilt treated with IMPLIX®

CONCLUSIONS

From the results of these experiments the following conclusions can be made : there is a good correlation between the two analytical methods.($r = 0,88$ ($n = 46$; $p \leq 0,001$). However, the gaschromatographic method yielded statistical significant lower androstenone concentrations than the MICROELISA method. This might be explained by differences in accuracy between the two methods or by differences in the length of the storage period of the samples . Since the samples for the gaschromatographic method were stored over a longer period (30 months versus 9 months for the ELISA method) the androstenone concentration may have decreased in function of storage time.

It is shown clearly that parenteral treatment of boars with REVALOR® significantly reduces the androstenone concentration of the backfat of the animals from a mean value of ca 0,5 ppm to a mean value of ca 0,1 ppm. The treatment of barrows with IMPLIX® and of gilts with REVALOR® or IMPLIX® does not significantly affect the already small androstenone level of backfat of these animals. In gilt fat samples no changes in the positional distribution of fatty acids within the triglycerides were found after treatment with REVALOR® or IMPLIX®

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