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Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry

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Abstract

The control on use of anabolic agents in meat producing animals is generally based on urine, faeces or hair analysis. This exercise, which is usually performed in slaughterhouses or on farms, is not relevant to imported carcasses or retail meat. A single sensitive method for a wide range of anabolic steroids was developed. After extraction of the lyophilised meat, enzymatic hydrolysis was used for deconjugation. Solid-phase extraction on a polymeric stationary phase was performed prior to hydrolysis of ester residues under alkaline conditions. Liquid–liquid partitioning was used to separate the analytes into two main categories: phenol containing molecules, such as phenolic steroids, resorcylic acid lactones and stilbenes, and Δ4-3-one containing molecules, such as most androgens and progestagens. Solid-phase extraction on silica columns was performed before applying a specific derivatisation for each compound sub-group. The combination of high-resolution chromatography with a quadrupole mass spectrometer permitted detection of 23 steroids in the 5–100 ng/kg range. Ion chromatograms for residue positive samples are shown and discussed.

Keywords: Food analysis; Steroids; Trenbolone; Melengestrol

1. Introduction

Natural and synthetic hormones have been used worldwide for many years to improve the rate of protein deposition in livestock. Although the use of steroids, or steroid-like compounds, is permitted and widely used in various countries, their use as growth promoters is prohibited in the European Union (EU). In spite of the official ban, a black market demand for hormones and hormone cocktails exists. Monitoring for illicit use of hormones is carried out under the terms of EU directive 96/23/EC [1] which is implemented through surveillance according to the National Plans of individual member states [2]. For controls at retail level, as well as for products imported into the EU, development of efficient methods applicable to meat samples are necessary. To establish the presence of anabolic agents and their metabolites in tissue samples, analytical techniques for detecting trace amounts of these compounds are required. The list of steroids liable to be abused in cattle fattening is ever-lengthening and the analytical requirements are increasingly stringent. For this reason, specific immunoassays [3–13], which allow the detection of only a single compound, are now being replaced by multiresidue techniques such as chromatography combined with mass spectrometry [14–22]. Apart from methods for the compounds permitted in various countries (melengestrol acetate, trenbolone acetate, zeranol, progesterone, oestradiol,

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testosterone and their esters), suitable analytical methods are those which allow detection of the main anabolic agents used illegally, such as nandrolone, methyltestosterone, norethandrolone, medroxyprogesterone and megestrol, at the low ng/kg level.

2. Experimental

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and provided by Merck (Darmstadt, Germany) and Solvants Documentation Synthesis (SDS, Peypin, France). *Helix pomatia* juice was from Biosepra (Villeneuve la Garenne, France). The solid-phase extraction (SPE) columns were from SDS (Harbor City, CA, USA) (silica: 1 g, aminopropyl: 0.5 g) and Supelco (St.-Quentin-Fallavier, France) (styrene–divinylbenzene copolymer resin, Envi-ChromP: 0.5 g). The derivatisation reagents *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), *tert*.-butyldimethylchlorosilane (TBDMCS) and trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Aldrich (Milwaukee, WI, USA). The reference steroids were from Steraloids (Wilton, NY, USA), Research Plus (Bayonne, NJ, USA) and RIVM (Bilthoven, Netherlands) (Table 1).

2.2. Tissue samples

Liver and muscle samples from untreated cattle were used as blank and, after fortification with the different steroids, as quality control samples. Steroid concentrations in these samples were at 0.5 ng/g. Commercial samples were collected by the Veterinary Inspection Services or were from animals

Table 1
List of screened analytes and their respective internal standards (retention times are only indicative, and are expressed in min)

Group	Analytes			Internal standards		
	Name	Ions	t_R (min)	Name	Ions	t_R (min)
Stilbenes	*cis*-DES	496/191	17.59			
	Hexestrol	249	19.12	[$^2H_4$]Hexestrol	251	19.08
	Dienestrol	494/479	19.23			
	trans-DES	496/191	19.38	[$^2H_6$]DES	502	19.32
RALs	Zeranol	538/433	18.91	[$^2H_4$]Zeranol	542	18.88
	Taleranol	538/433	19.08	[$^2H_4$]Taleranol	542	19.05
Estrogens	17α-Oestradiol	416/285	17.30			
	17β-Oestradiol	416/285	17.89	[$^2H_3$]17β-oestradiol	419	17.87
Androgens	17α-Trenbolone	449/380	15.13			
	17β-Trenbolone	442/380	15.26	[$^2H_2$]17β-trenbolone	444	15.25
	17α-Nandrolone	418/194	16.70			
	17α-Testosterone	432/209	17.01			
	17β-Nandrolone	418/194	17.30	[$^2H_3$]17β-nandrolone	421	17.27
	17β-Testosterone	432/209	17.63	[$^2H_2$]17β-testosterone	434	17.61
	Norethandrone	432/287	18.42			
	Bolasterone	460/445	18.64			
	Methyltestosterone	446/301	18.75	[$^2H_3$]Methyltestosterone	449	18.72
	Ethinyltestosterone	456/301	18.97			
	Norethandrolone	446/287	19.97			
Gestagens	Progesterone	458/443	20.35	Norprogesterone	444	20.06
	Megestrol	558/453	20.92	[$^2H_3$]Megestrol	561	20.88
	Chlormadinone	578/473	21.03			
	Medroxyprogesterone	560/330	21.07	[$^2H_3$]Medroxyprogesterone	563	21.03
	Melengestrol	570/555	21.17	[$^2H_3$]Melengestrol	573	21.14

treated with anabolic steroids or associated compounds. After collection, tissue samples were stored frozen, below −18°C.

2.3. Extraction and purification procedure

2.3.1. Preparation

Tissue sample (15 g) was lyophilised and ground to a powder. Liquid–liquid extraction (LLE), using 12 ml methanol and 15 ml acetate buffer (0.2 *M*, pH 5.2), gave extraction of all forms of the compounds (esters, free and conjugates). After evaporation of the methanol, 80 μl *Helix pomatia* (β-glucuronidase 10^5 Fischman U ml^{-1} and sulfatase 10^6 Roy U ml^{-1}) were added to the remaining acetate buffer and the extract was incubated for at least 15 h at 52°C and pH 5.2. Following deconjugation, the extract was transferred to a column reservoir and passed through the copolymeric phase SPE column (ENVI-ChromP), which had been previously conditioned with 6 ml ethyl acetate, 6 ml methanol and 6 ml water. After elution of interfering compounds with 3 ml hexane, the analytes were eluted from the SPE column with 14 ml hexane–diethyl ether (70:30, v/v). After evaporation, ester forms were hydrolysed for 30 min at 50°C with 500 μl sodium methylate (1% w/v in methanol). Then, addition of 1 ml sodium hydroxide (1 *M*) and 8 ml hexane–diethyl ether (70:30, v/v) allowed the separation of phenolic (stilbenes, resorcylic acid lactones and phenolic steroids) and Δ4-3-one compounds (androgens and progestagens) into the aqueous phase and organic phase, respectively (Fig. 1).

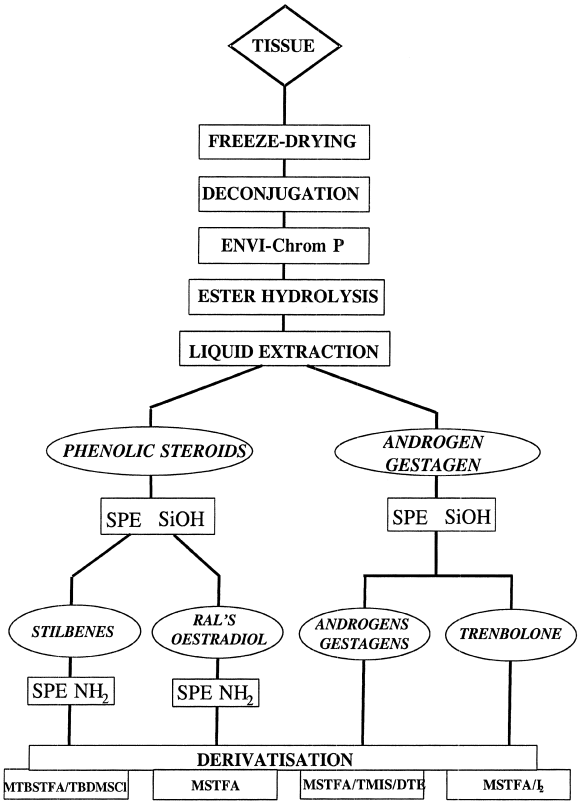


Fig. 1. General analytical procedure (SPE: solid-phase extraction, LLE: liquid–liquid extraction).

2.3.2. Purification of phenolic steroids, resorcyclic acid lactones and stilbenes

The pH of the aqueous phase was neutralised by the addition of 150 μl glacial acetic acid and then 8 ml diethyl ether was used to extract the analytes. After removal and evaporation of the diethyl ether, the dry residue was dissolved in 500 μl hexane–dichloromethane (60:40, v/v) and applied to a SPE silica column which had been conditioned with hexane. After elution of interfering compounds with 3 ml hexane–ethyl acetate (75:25, v/v), stilbenes were eluted with 13 ml hexane–ethyl acetate (85:15, v/v) and resorcylic acid lactones and phenolic steroids were eluted with 13 ml hexane–ethyl acetate (60:40, v/v). The two fractions were dried and reconstituted in 2 ml ethyl acetate–methanol (80:20, v/v).

Each fraction was applied to aminopropyl SPE columns previously conditioned with 4 ml ethyl acetate–water (25:0.68, v/v) and 4 ml ethyl acetate–methanol (80:20, v/v). The analytes were not retained by the stationary phase and elution was completed with an additional 2 ml volume of ethyl acetate–methanol (80:20, v/v).

2.3.3. Purification of Δ4-3-one compounds

After evaporation of the organic phase (2.3.1), the dry residue was dissolved in 500 μl hexane–dichloromethane (60:40, v/v) and applied to a SPE silica column, previously conditioned with hexane. The column was washed with 8 ml hexane–ethyl acetate (75:25, v/v). The analytes were eluted with 13 ml hexane–ethyl acetate (60:40, v/v) and the

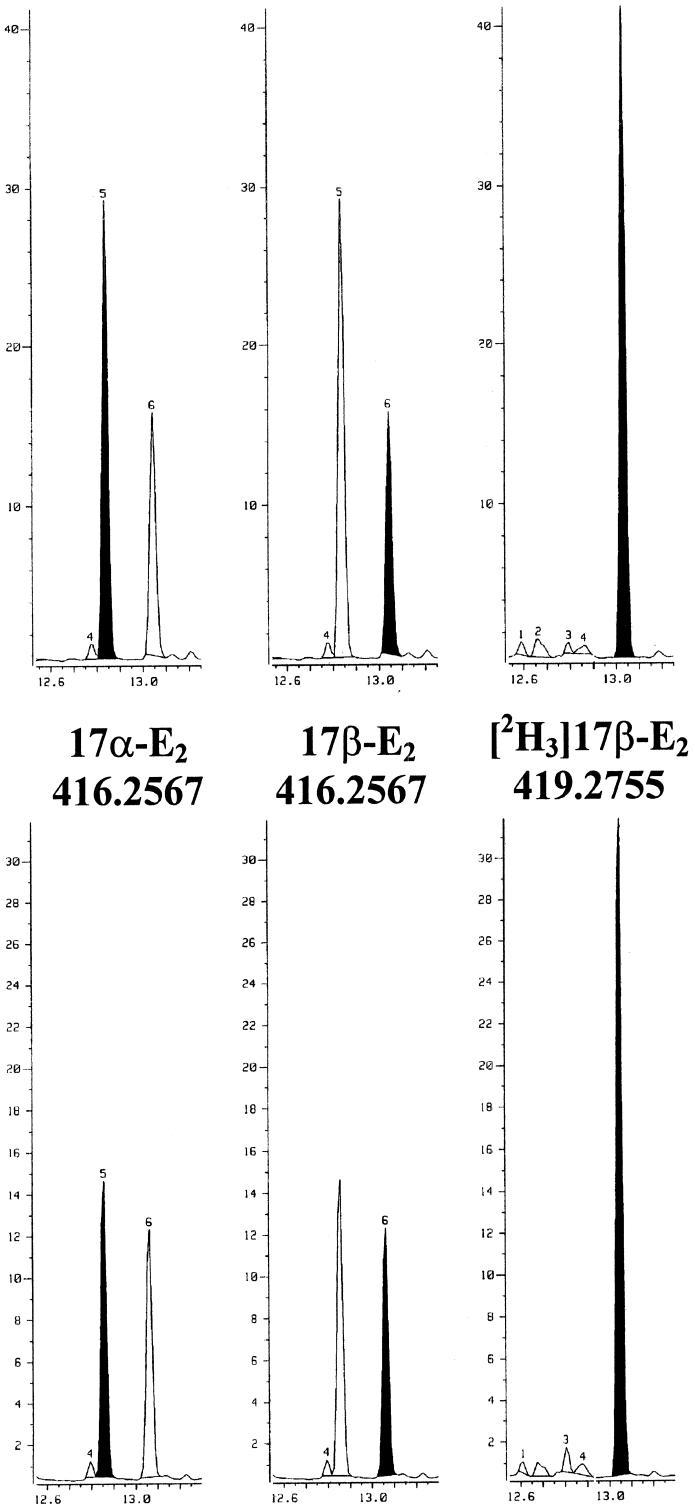


Fig. 2. Ion chromatograms corresponding to 17α-oestradiol, 17β-oestradiol and [$^{2}H_{3}$]17β-oestradiol (from left to right) extracted from an incurred meat sample, deconjugated (above) or not (below) with *Helix pomatia*.

eluate was divided in two, representing (a) trenbolone metabolites and (b) androgens and progestagens.

2.4. Derivatisation

2.4.1. Δ4-3-one compounds (except trenbolone metabolites)

Fifteen μl of MSTFA–TMIS–DTT (1000:5:5, v/v/w) were applied to the dry residue and incubated for 40 min at 60°C.

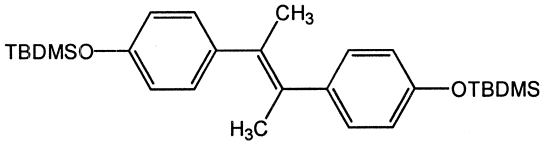
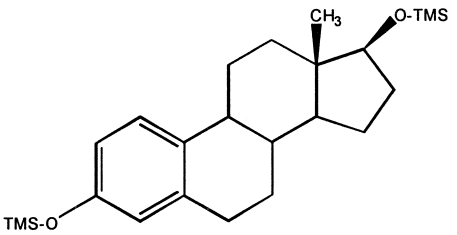
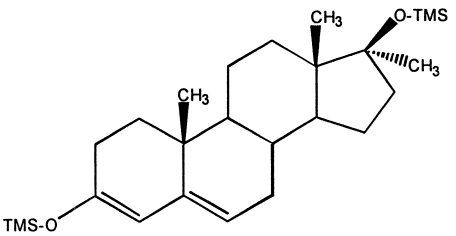
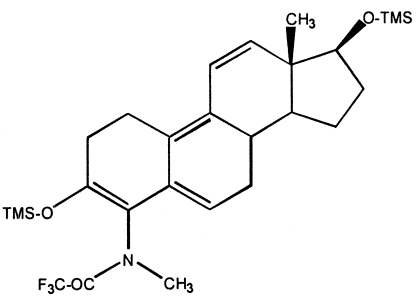
2.4.2. Trenbolone metabolites

Ten μl of MSTFA–I_2 (1000:4, v/v) were applied to the dry residue; the reaction occurs instantaneously at ambient temperature.

2.4.3. Resorcylic acid lactones and phenolic steroids

Fifteen μl of MSTFA were applied to the dry residue and total derivatisation of the alcohol functions was obtained by incubation for 40 min at 60°C.

Table 2
Derivatised analytes depending on the derivatisation reagent used

Groups	Reagent	Structure
Stilbenes	MTBSTFA–TBDMCS	
Oestrogens-RAL's	MSTFA	
Andro-gestagens	MSTFA–TMIS–DTT	
Trenbolone	MSTFA–I_2	

2.4.4. Stilbenes

Fifteen μl of MTBSTFA–TBDMCS (100:1, v/v) were applied to dry residue; and incubated for 60 min at 75°C.

2.5. GC–MS analysis

The quadrupole MS system used was a model 5973 coupled to a model 6890 gas chromatograph, both from Hewlett-Packard (Palo Alto, CA, USA). The split/splitless injector was maintained at 250°C, the pulse time for the splitless mode was set at 1.5 min, and the injected volume was 3 μl. The column used for separation of trenbolone metabolites was a MN-δ3 from Macherey–Nagel. Otherwise, an OV-1 (Ohio-Valley) was used. Both had the same characteristics (30 m×0.25 mm I.D., film thickness 0.25 μm). The GC parameters used on the MN-δ3 column were 120°C (2 min), 15°C min^{-1} until 300°C (5 min). The GC parameters used on the OV-1 column

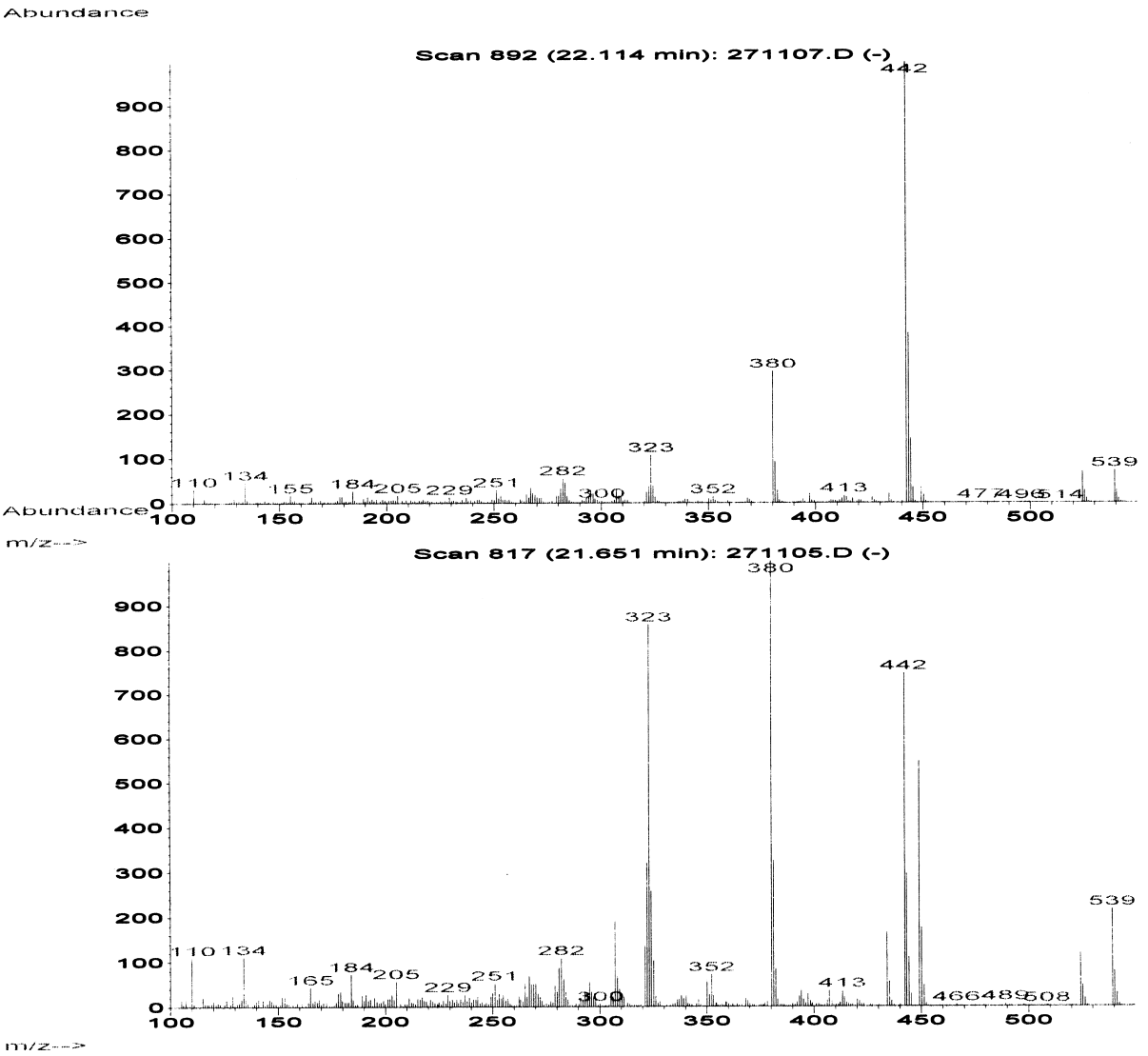


Fig. 3. Mass spectra of 17α- (above) and 17β-trenbolone (below) (MSTFA–I_2 derivatisation, EI ionisation, full mass spectrum).

were 120°C (2 min), 15°C min^{-1} until 250°C (0 min), then 5°C min^{-1} until 300°C (8 min). Helium (N55) was used as carrier gas at 1 ml min^{-1}. The electronic beam energy on the mass spectrometer was set at 70 eV in the electron impact (EI) mode.

3. Results and discussion

3.1. Tissue preparation

Freeze-drying was preferred to enzymatic digestion [18,25], matrix solid-phase dispersion [24] or solvent extraction of the fresh sample. Lyophilisation permits grinding of the sample to a fine powder, resulting in increased interaction between sample and solvent. Better extraction yield and more efficient deconjugation are achieved.

3.2. Deconjugation

Enzymatic hydrolysis of steroid glucuronides or sulfates in muscle using *Helix pomatia* juice is not used by all scientists involved in this field. However, a significant proportion of steroid residues are glucurono- or sulpho-conjugated, and sometimes linked to glycosyl groups. The total quantity of 17α- and 17β-oestradiol determined with or without *Helix pomatia* deconjugation was evaluated.The two chromatograms in Fig. 2 show that deconjugation gives an increase of approximately 50% in the yield of 17α-oestradiol but only a slight increase of approximately 5% in the case of 17β-oestradiol; this observation indicates that 17α-oestradiol is probably more conjugated than 17β-oestradiol. The conditions used for deconjugation were those published elsewhere [26–31].

3.3. Extraction on copolymeric phase

A copolymeric phase combining styrene and divinylbenzene groups was substituted for the commonly used octadecylsilyl stationary phase. Hexane–diethyl ether was sufficient for elution of the weakly retained target analytes. The eventual chromatographic separations obtained were better with this polymer than with C_{18}, C_8 or CH phases, which require methanol elution because of the non-elution of strongly retained interfering compounds.

3.4. Hydrolysis of esters

Some of the anabolic agents used in livestock

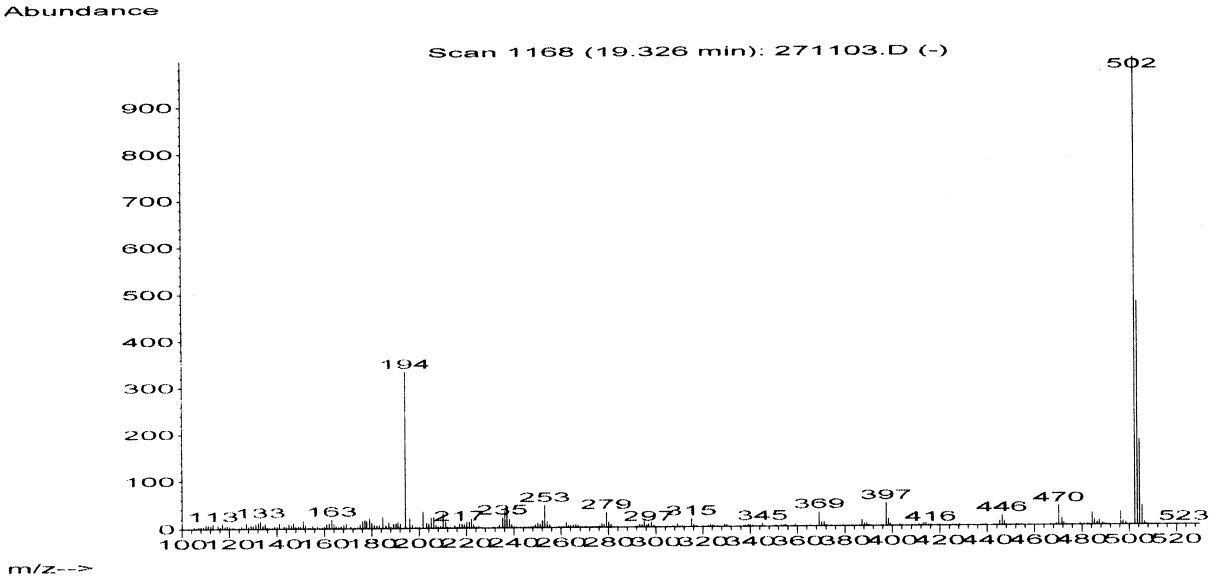


Fig. 4. Mass spectrum of [$^2H_6$]diethylstilbestrol (MTBSTFA–TBDMCS derivatisation, EI ionisation, full mass spectrum).

production, such as esters of progestagens, occur unchanged in tissues. In particular, the acetate form of melengestrol, megestrol, medroxyprogesterone or chlormadinone, are well known to be stable, and are not hydrolysed by plasmatic esterase. The target residue is, therefore, the ester but these molecules are difficult to analyse by GC–MS, with degradation probably occurring in the injector liner or on the column. Consequently, we developed a hydrolysis step to convert the acetates into the corresponding free alcohols. Sodium methylate (1%, w/v, in methanol) at 50°C for 30 min was found to be suitable, giving a comparable response, generally, to that obtained with sodium hydroxide–ethanol and, for some compounds like progestagens, giving a response up to two times better.

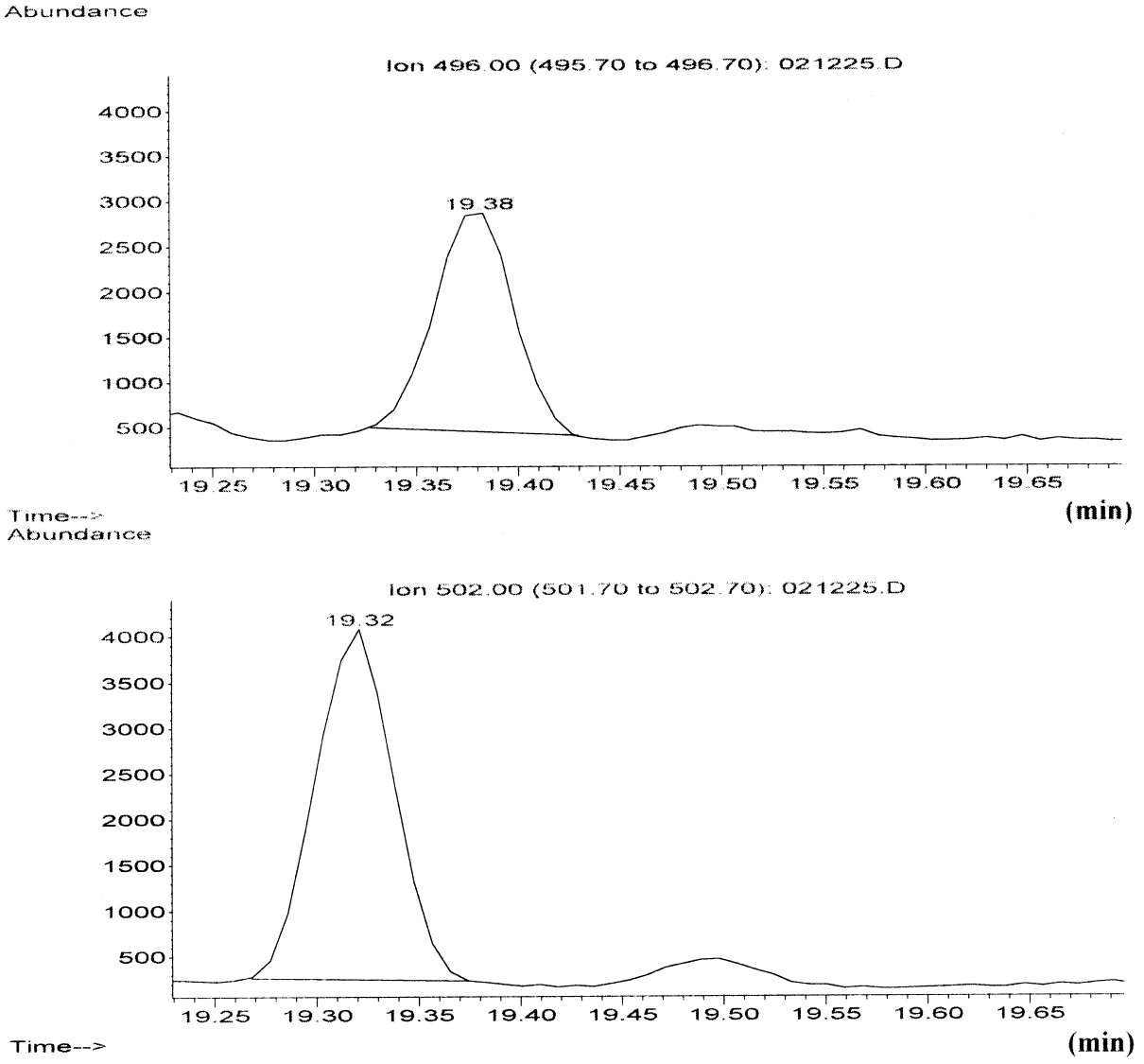


Fig. 5. Ion chromatograms of a meat sample fortified with 200 ng/kg diethylstilbestrol (m/z 496, 19.38 min) and 500 ng/kg of [$^2H_6$]diethylstilbestrol (m/z 502, 19.32 min) (MTBSTFA–TBDMCS derivatisation, EI ionisation, SIM chromatogram).

3.5. Separation of phenolic and Δ4-3-one compounds

Because ion chromatograms are particularly difficult to interpret for endogenous androgens and oestrogens, it was desirable to isolate one group from the other. A fast and cheap option based on liquid–liquid partitioning was used. Addition of sodium hydroxide to non-polar solvents, such as hexane or diethyl ether, converts all phenol-containing analytes into phenolate forms. As a result, these analytes remain in the aqueous phase, while non-phenolic steroids, because of their low polarity, transfer into the organic phase, resulting in much cleaner ion chromatograms.

3.6. Purification of phenolic compounds

A chromatographic (SPE) system combining hexane–ethyl acetate as mobile phase (with an increas-

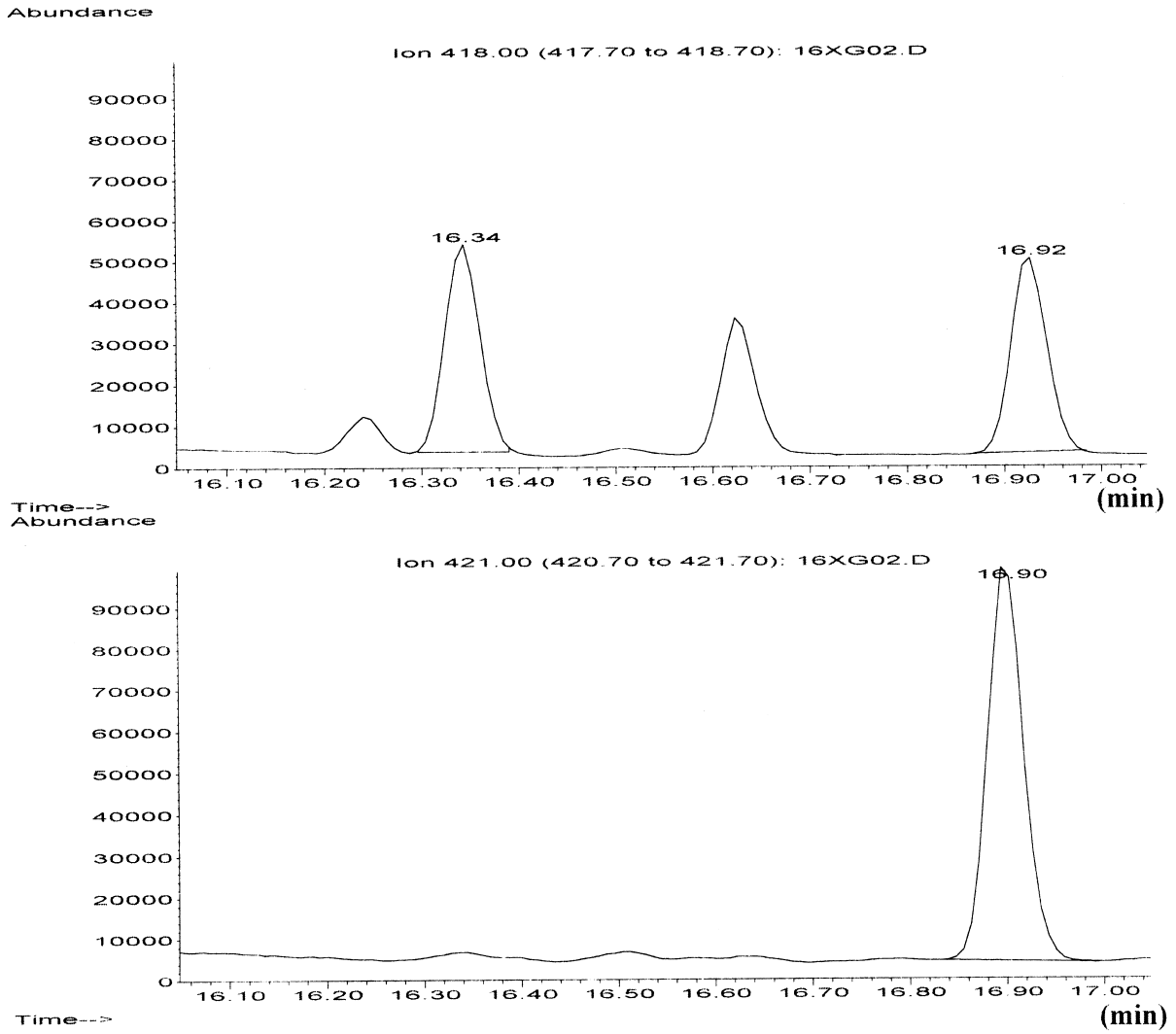


Fig. 6. Ion chromatograms of a meat sample fortified with 200 ng/kg 17α-nandrolone, 17β-nandrolone (*m/z* 418, 16.34 min and 16.92 min) and 500 ng/kg of [$^2H_3$]17β-nandrolone (*m/z* 421, 16.90 min) (MSTFA–TMIS–DTT derivatisation, EI ionisation, SIM chromatogram).

ing gradient of ethyl acetate) with silica as stationary phase was found suitable for phenolic compounds purification [23]. Because of their low polarity, stilbenes were eluted with hexane–ethyl acetate (85:15, v/v), while hexane–ethyl acetate (60:40, v/v) was required for elution of resorcylic acids lactones and oestradiol. Each group was further purified on a disposable aminopropyl column. Ethyl acetate–methanol (80:20, v/v) was used to load the sample onto the column and to elute the analytes. This fast step gave appreciable purification in term of ion chromatogram interpretation without affecting the recovery yield.

3.7. Purification of Δ4-3-one compounds

The same approach was applied to androgen and progestagen compounds. The only difference was in the composition of the mobile phase used for eluting these analytes. Being slightly more polar than resorcylic acid lactones and oestradiol, hexane–ethyl acetate (60:40, v/v) was used for elution and gave a suitably clean extract for analysis by GC–MS.

3.8. Derivatisation

A different derivatisation procedure was applied to each group of steroids. The structures of the derivatised products are given Table 2. All androgens and progestagens were derivatised using MSTFA–TMIS–DTT, the most popular derivatisation procedure used in steroid residue analysis. Ketone and alcohol functions are converted into enol-trimethylsilyl and ether-trimethylsilyl groups, respectively.

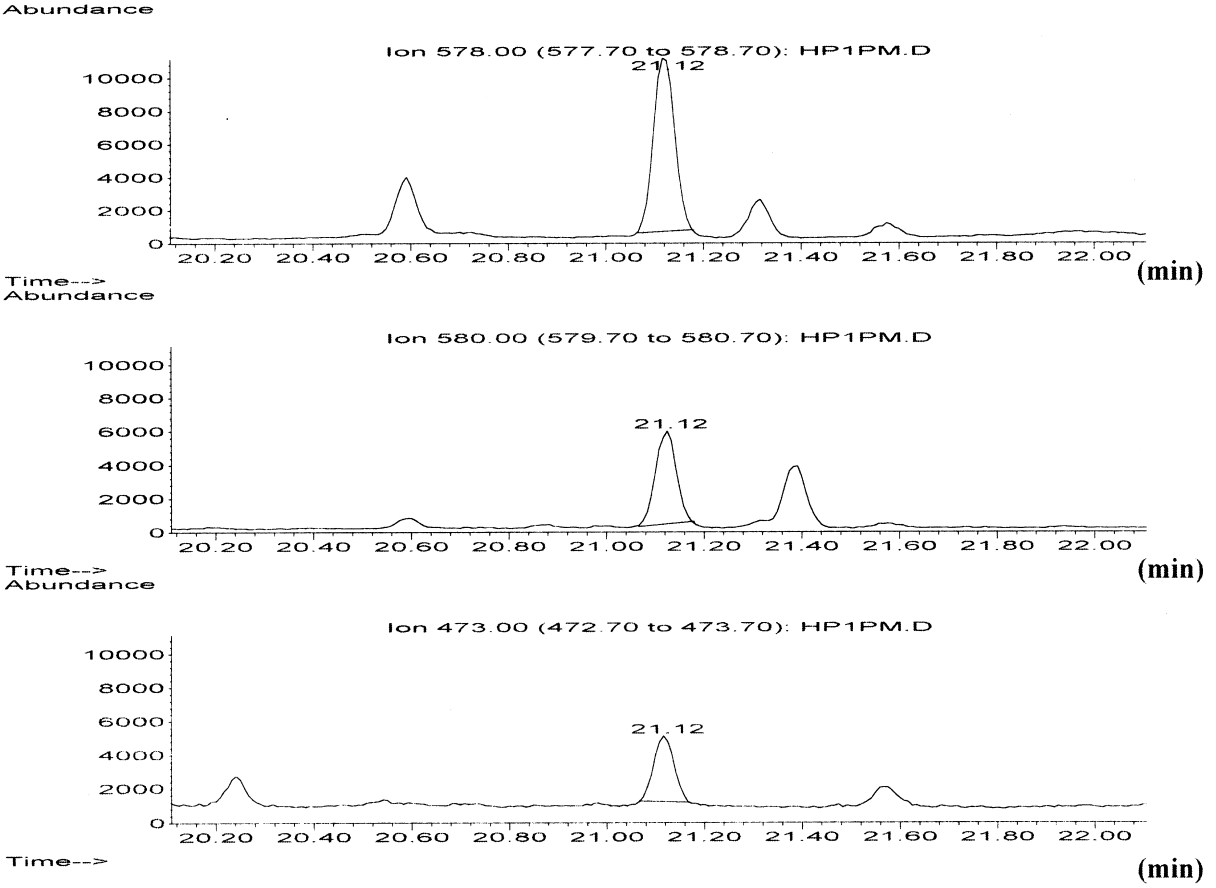


Fig. 7. Ion chromatograms of a chlormadinone acetate positive meat sample (m/z 580, 578, 473, 21.12 min). Estimated concentration: [2.5 μg/kg] (MSTFA–TMIS–DTT derivatisation, EI ionisation, SIM chromatogram).

DTT is necessary to prevent any degradation of the silylating reagent, caused by iodide oxidation, although it may cause intense interferences in the ion chromatograms. Trenbolone and its derivatives are well-known exceptions within the androgen group. Use of the MSTFA–TMIS–DTT derivatisation reagent for trenbolone results in no response in gas chromatography. The TMS-enol, TMS-ether derivative is not observable in GC–MS, either because of the three conjugated double bond structure which affects reactivity or because of thermal instability of the derivative in the gas chromatograph. Therefore, we developed an alternative derivatisation based on the action of MSTFA in combination with I_2 [32]. The main product formed was by TMS-enolisation of the 3-keto group, silylation of the 17-hydroxyl group and addition of $N(CH_3)COCF_3$ to the steroid nucleus (Table 2). This derivative possesses very good GC properties and interesting fragmentation in the electron impact ionisation mode. Moreover, the mass spectrum of the two epimeric forms of trenbolone (17α- and 17β-hydroxyl) display large differences, allowing differentiation of the two isomers by their mass spectra (Fig. 3). The derivatisation used for resorcylic acid lactones and oestradiol-like compounds was based on MSTFA without any catalyst. Since only aliphatic and aromatic alcohols have to be derivatised, a moderately powerful silylating reagent is sufficient. Stilbenes, also, may be derivatised using MSTFA but, because of their relatively high volatility, the retention times of the TMS derivatives at less than 12 min using the specified GC conditions. The main consequences are possible interfering signals with early eluting compounds, which can interfere

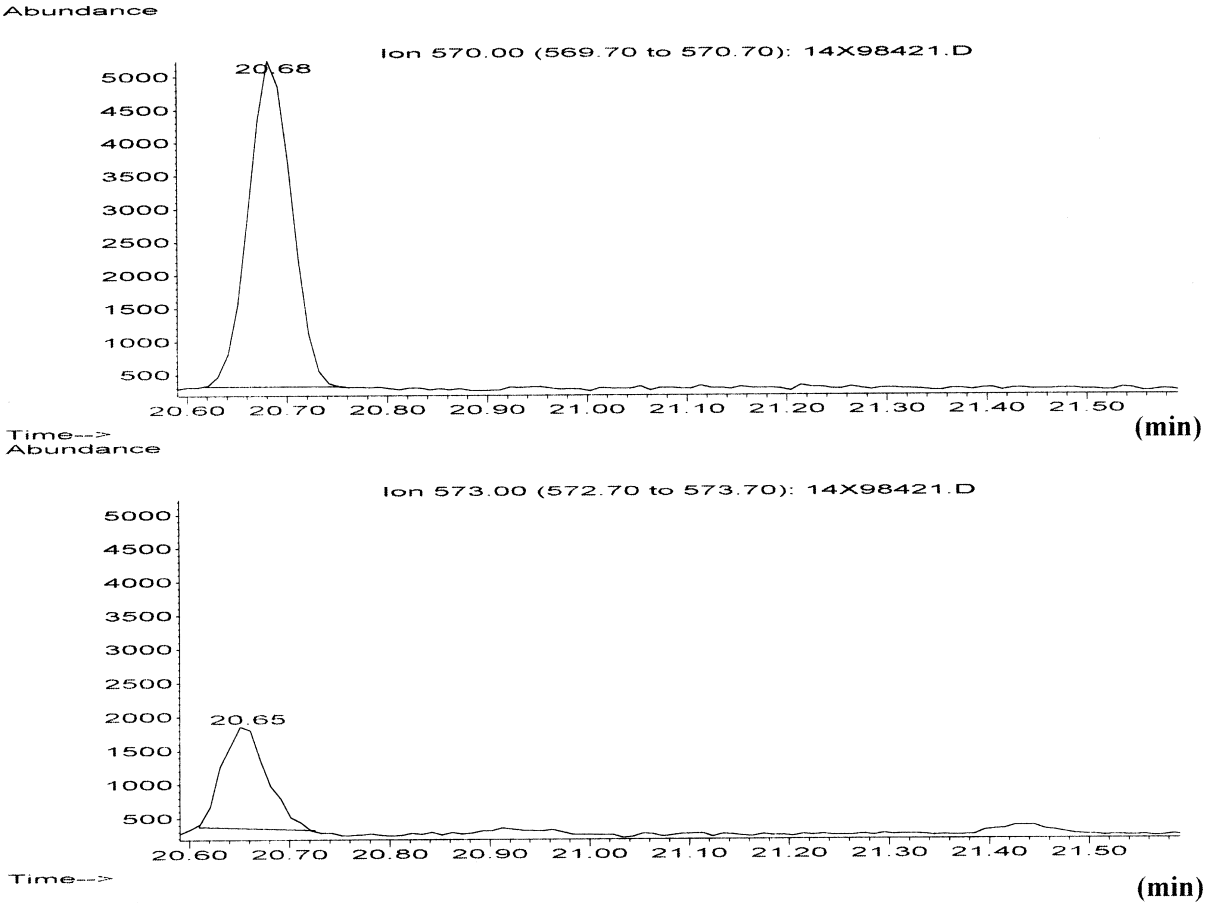


Fig. 8. Ion chromatograms of a suspected melengestrol acetate positive meat sample (melengestrol m/z 570, 20.68 min; [$^2H_3$]melengestrol m/z 573, 20.65). Estimated concentration: [1.5 μg/kg] (MSTFA–TMIS–DTT derivatisation, EI ionisation, SIM chromatogram).

with ion chromatogram interpretation. Formation of di-TBDMS derivatives increases greatly stilbene retention times and the mass to charge ratio of the diagnostic ions, so that the ion chromatograms become more specific. The mass spectrum of [$^2H_6$]diethylstilbestrol can be seen in Fig. 4. The relative stability of this derivative results in only a moderate fragmentation and a high relative intensity of the molecular ion (*m*/*z* 418, base peak).

3.9. Ion chromatograms for fortified samples

Characteristic ion chromatograms for each group of molecules are shown in Figs. 5 and 6. Blank meat or liver samples were fortified at different levels depending on the analytes being measured. Fig. 5 shows the signals for 200 ng/kg *trans*.-diethylstilbestrol (*m*/*z* 496, 19.38 min) and 500 ng/kg of *trans*.-[$^2H_6$]diethylstilbestrol (*m*/*z* 502, 19.32 min) in a meat sample. The molecular ion chromatogram is very specific; the measured signal-to-noise ratio is 30. Fig. 6 shows an ion chromatogram (*m*/*z* 418) for a meat sample fortified with 17α- and 17β-nandrolone, at 200 ng/kg, as well as one for 17β-[$^2H_6$]nandrolone (*m*/*z* 421), their internal standard. The analyte signals are not disturbed by any interferences at the retention times; the signal-to-noise ratio at this concentration is estimated to be 200.

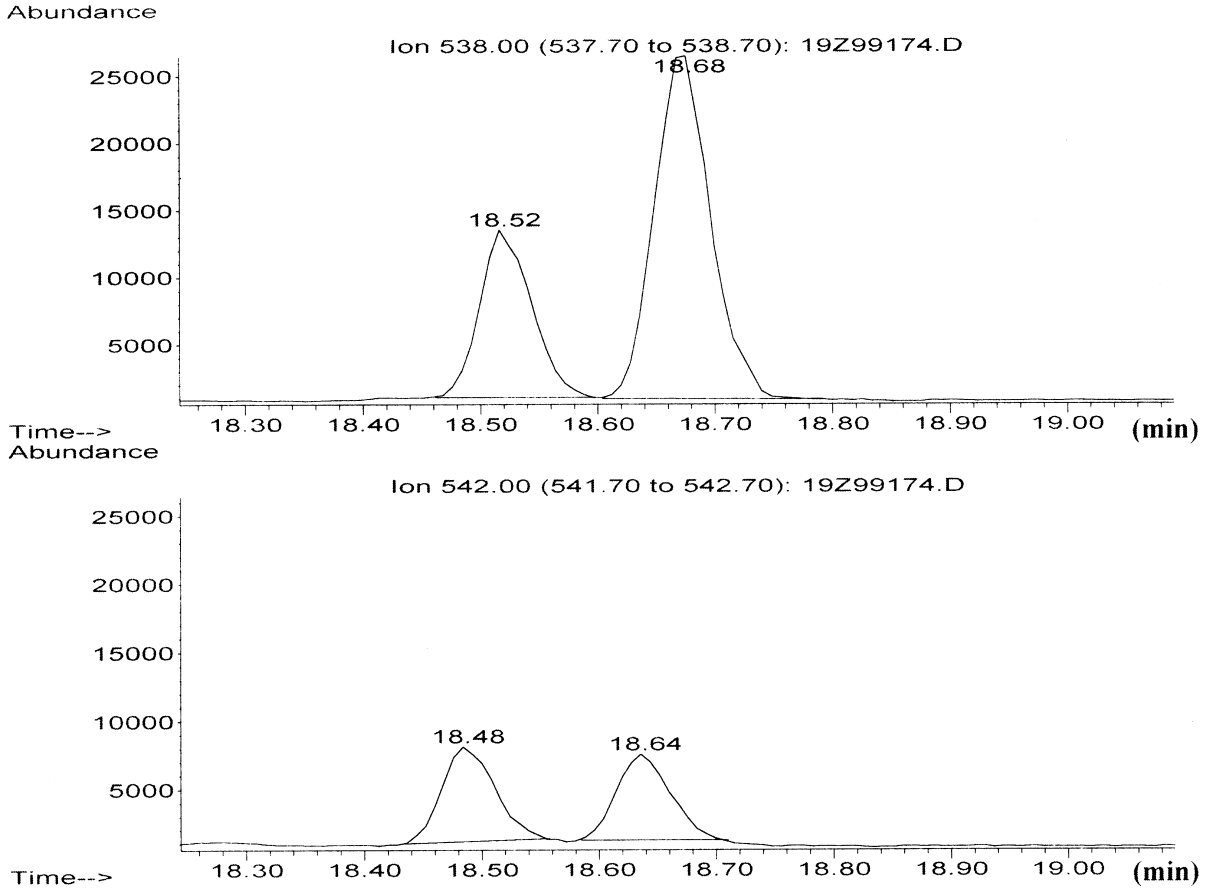


Fig. 9. Ion chromatograms of a suspected zeranol/taleranol positive liver sample (*m*/*z* 538, zeranol 18.52 min and taleranol 18.68 min; *m*/*z* 542, [$^2H_4$]zeranol 18.48 min and [$^2H_4$]taleranol 18.64 min). Estimated concentration: zeranol=[1.2 μg/kg] and taleranol=[0.9 μg/kg] (MSTFA derivatisation, EI ionisation, SIM chromatogram).

3.10. Ion chromatograms for incurred samples

Figs. 7–10 show chromatograms for residue positive meat and liver samples from animals treated with anabolic agents. Fig. 7 shows ion chromatograms for a chlormadinone-positive meat sample taken from an animal which received an intramuscular injection of chlormadinone acetate (250 mg) and was slaughtered one month later. Three diagnostic ions were monitored, the molecular ion (m/z 578), the ion due to the ^{37}Cl isotope (m/z 580) and an ion corresponding to the loss of $COCH_2OTMS$ at the 17 position (m/z 473). All ions satisfied EU analytical criteria, in terms of correspondence of retention times and ion ratios with standards [1]. Concentration was estimated to be 2.5 μg/kg. Ion chromatograms for a suspect meat sample, taken by the official veterinary inspection services, are shown in Fig. 8. The m/z 570 ion chromatogram demonstrates the presence of melengestrol and the concentration was determined to be 1.5 μg/kg. A liver sample, suspected as containing zeranol and its metabolite taleranol, showed the presence of the two metabolites (Fig. 9). Concentrations of zeranol and taleranol were estimated to be 1.2 and 0.9 μg/kg, respectively. Fig. 10 shows the ion chromatograms for a

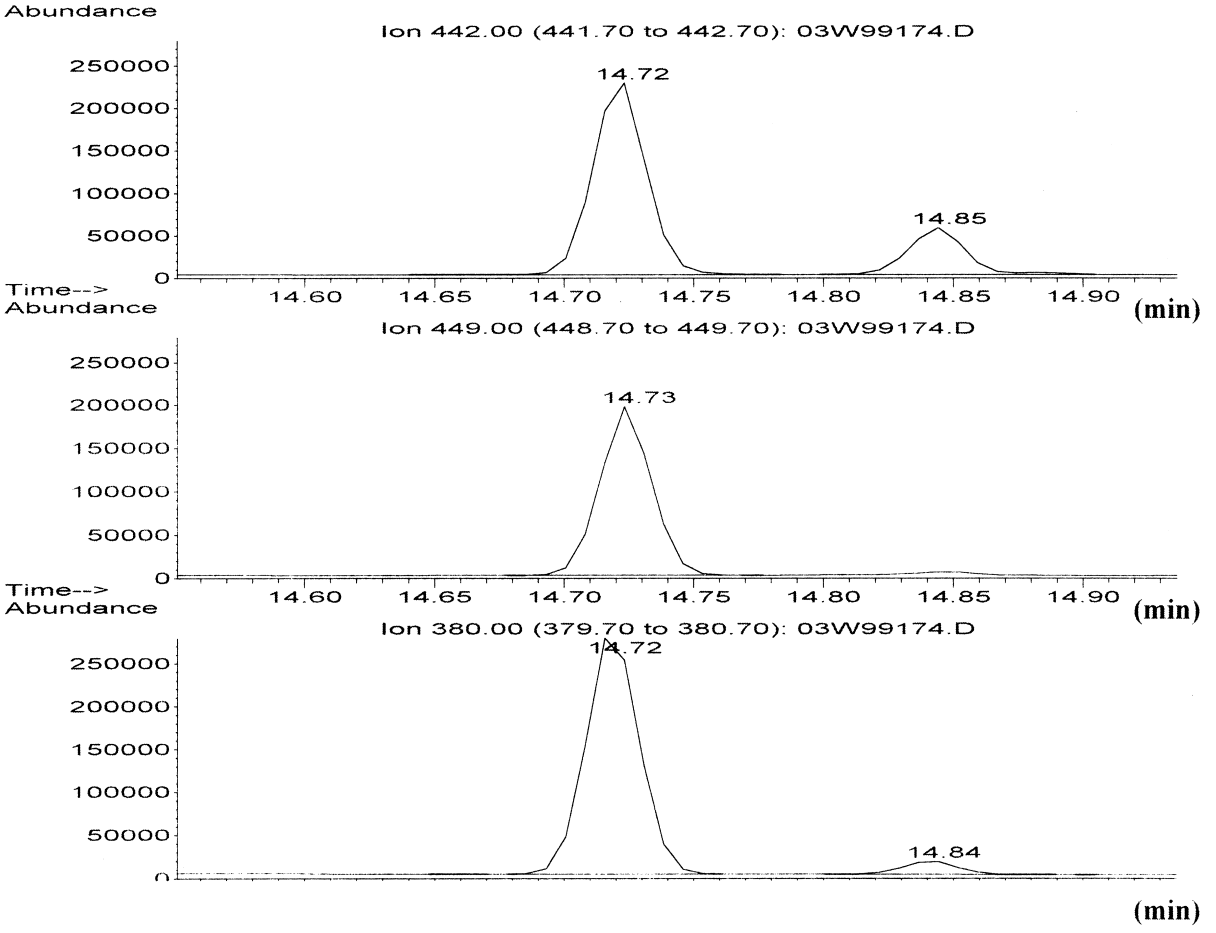


Fig. 10. Ion chromatograms of a suspected trenbolone positive liver sample (17α- and 17β-trenbolone m/z 442, 449, 380, 14.73 min and 14.85 min). Estimated concentration: 17α-trenbolone=[9.0 μg/kg] and 17β-trenbolone=[1.5 μg/kg] (MSTFA–I_2 derivatisation, EI ionisation, SIM chromatogram).

suspect liver sample. Three diagnostic signals corresponding to 17α-trenbolone and 17β-trenbolone were detected. Retention times and relative ion ratios for the samples corresponded to those for standard molecules and concentrations of 9.0 and 1.5 μg/kg were determined for 17α-trenbolone and 17β-trenbolone, respectively. The presence of the added radical $N(CO)CF_3$, coupled with high-resolution measurements, allows identification and quantification of trenbolone metabolites at very low concentrations [32,33].

3.11. Confirmatory analysis

The developed procedure was applied on a low-resolution mass spectrometer. However, because of the low ng/kg levels of anabolic steroid residues in meat, unambiguous identification of analytes required, in most cases, use of high resolution on an electromagnetic mass filter instrument (reverse geometry, Matsuda type) [32,33]. The minimum criteria applied were those of European Decision 93/256/EC in terms of correspondence of the retention time for the suspect analyte with that for the standard and MS parameters such as signal to noise, relative ratios of four diagnostic ions being within the ±10% tolerance, etc.

4. Conclusion

A specific and sensitive method for testing for a large number of anabolic steroids at low ng/kg levels has been described. Validation was carried out on fortified samples and the method was evaluated further on chlormadinone acetate, melengestrol acetate, trenbolone acetate and zeranol treated animals. The corresponding analytes were unambiguously detected in meat and/or liver samples. Finally, the method was tested on 600 routine samples and its ruggedness was demonstrated.

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