The use of supercritical fluid extraction for the determination of steroids in animal tissues

Alida A. M. Stolker,* Paulus W. Zoontjes and Leendert A. van Ginkel

[a] *National Institute of Public Health and the Environment, Laboratory for Residue Analysis, PO Box 1, 3720 BA Bilthoven, The Netherlands*

Received 2nd July 1998, Accepted 2nd October 1998

A multi-analyte, multi-matrix method was developed for the routine determination of steroids in animal tissues (skin, meat and fat). After addition of internal standards and sample pre-treatment, the analytes of interest were extracted from the matrix with unmodified supercritical CO_2 and trapped directly on an alumina sorbent placed in the extraction vessel (in-line trapping under supercritical conditions). After extraction, alkaline hydrolysis was performed and the analytes were derivatised. The samples were then analysed by gas chromatography-mass spectrometry. The limit of detection for the different matrix–analyte combinations was 2 μg kg^{-1} (for melengestrol acetate 5 μg kg^{-1}), the repeatability ranged from 4 to 42% (n = 9) and the reproducibility ranged from 2 to 39% (n = 3).

Introduction

Throughout the European Union, the use of anabolic steroids is prohibited in food producing animals. Analytical methods based on liquid–liquid extraction (LLE), solid phase extraction (SPE), high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS)[1] are available to control the illegal use of this large group of compounds. The procedures, however, often are time and organic solvent consuming and the methods have to be optimised for every new steroid–matrix combination. The availability of a multi-analyte, multi-matrix extraction method is essential for the development of quick and efficient methods for the determination of anabolic steroids.

Supercritical fluid extraction (SFE) has shown great potential in offering shorter extraction times with high recoveries and low consumption of organic solvents. An overview of SFE applications for the extraction of residues of veterinary drugs and growth promoting agents from food and biological matrices was given by Stolker *et al*.[2] Din *et al*.[3] described the extraction of the anabolic steroid trenbolone from beef with modified supercritical CO_2. Huopalahti and Henion[4] described the SFE of seven estrogenic and anabolic agents from bovine tissues. Most samples of tissue contain various levels of fat, which, because of its relatively high solubility in supercritical CO_2, is co-extracted and can give problems during the final chromatographic analysis (HPLC or GC). Co-extraction of fat makes it necessary to perform a post-SFE clean-up procedure. By using a combined technique of supercritical fluid and solid phase extraction (SFE-SPE; Fig. 1) it has proved possible to extract residues of steroids from samples of animal tissue at residue levels.[5] The SFE-SPE technique was also used by Parks *et al*.[6] for the extraction of melengestrol acetate from bovine kidney fat. They used the in-line trapping technique (trapping under supercritical conditions on a sorbent which is situated in the extraction vessel). By choosing the right conditions, the fat was not retained on the sorbent in the extraction vessel, in contrast to the analytes of interest. The final analysis was by GC with mass-selective detection. A key limitation of SFE for residue analysis is that the sample size may be too small to achieve the required limits of detection. By freeze-drying the samples of tissue before the SFE, this problem can be overcome.

By choosing the right combination of freeze-drying, SFE and SPE (Fig. 2), a multi-extraction technique has become available which can be used in routine residue determinations of steroids in samples of animal tissues such as meat, skin and fat.

Experimental

Chemicals

The standards of the steroids (Table 1) were obtained commercially or were gifts from the pharmaceutical industry. The deuterated analogues were synthesized in-house (RIVM, Bilthoven, The Netherlands). The samples of bovine kidney fat, muscle and skin were collected at Dutch slaughterhouses. Methanol, acetone, ethanol, *tert*-butyl methyl ether (TBME), sodium hydroxide, hydrochloric acid (37%), acetic acid, potassium hydroxide, light petroleum, tris(hydroxymethyl)aminomethane (TRIS) and Extrelut were obtained from Merck (Amsterdam, The Netherlands). Subtilisin A, aluminium oxide (neutral, activity grade 1, type WN-3), dithioerythritol, ammonium iodide and polypropylene wool were obtained from

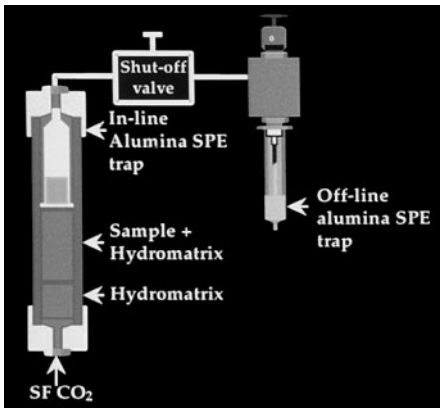

Fig. 1 SFE system configured for off-line analyte collection using a standard 6 ml SPE column and in-line analyte collection using a Teflon sleeve fitted with a standard 3 ml SPE column.

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

Sigma-Aldrich (Steinheim, Germany). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Alltech Nederland (Breda, The Netherlands). Heptafluorobutyric anhydride (HFBA) was a Pierce product (supplied by Omnilabo, Breda, The Netherlands). CO_2 of SFC/SFE grade without helium head pressure was obtained from Air Products (Waddinxveen, The Netherlands).

Stock standard solutions of the steroids (1 mg ml^{-1}) were prepared in methanol and stored at −20 °C. Working standard solutions were prepared by a series of 10-fold dilutions of the 1 mg ml^{-1} solutions. These solutions were stored in the dark at approximately 4 °C (range 1–10 °C) for a maximum period of 6 months. Internal standard solutions containing the steroids at a concentration of 1 ng μl^{-1} in methanol were prepared from the working standard solutions. A 0.1 mol l^{-1} TRIS buffer elution of pH 9.5 was prepared by dissolving 12.1 g of TRIS in 800 ml of water. After adjusting the pH to 9.5 ± 0.1, water was added to a final volume of 1000 ml. The 2 mol l^{-1} acetate buffer solution of pH 5.2, used to adjust the pH of samples of urine, was prepared by dissolving 25.2 g of acetic acid and 129.5 g of sodium acetate in 800 ml of water. After adjusting the pH to 5.2 ± 0.1, water was added to a final volume of 1000 ml. The solution for alkaline hydrolysis was prepared by dissolving 5.6 g of potassium hydroxide in 100 ml of methanol. An acidic buffer used to stop the hydrolysis was obtained by mixing 1.7 ml of hydrochloric acid (37%) with 98.3 ml of acetate buffer (2 mol l^{-1}).

Apparatus

An Epsilon 2 freeze-drier, was obtained from Christ (Östenrode, Germany). An Applied Separations Spe-ed 680 SFE system was obtained from Applied Science Group (Emmen, The Netherlands). The instrument is configured for the parallel extraction of two 24 ml vessels. The 3 ml disposable polypropylene extraction columns used for in-line trapping were obtained from Baker (Emmen, The Netherlands). Teflon sleeves were used to retain the 3 ml SPE columns in the SFE extraction vessels (Applied Science Group). The GC-MS system, obtained from Hewlett-Packard (Amstelveen, The Netherlands), consisted of a Model 5890 Series II Plus gas chromatograph, Model 7673 autoinjector and Model 5972 mass-selective detector. The steroids were separated on a CP-SIL 5CB column (25 × 0.25 mm id, film thickness 0.12 μm) (Chrompack, Bergen op Zoom, The Netherlands).

Sample preparation

Muscle. From the laboratory sample, a test portion of 5.0 g of tissue was freeze-dried. The freeze-dried sample powder was directly blended with 3.5 g of Extrelut. Internal standard solutions (25 μl of 1.0 ng μl^{-1} standard solution) and 1 ml of water were added.

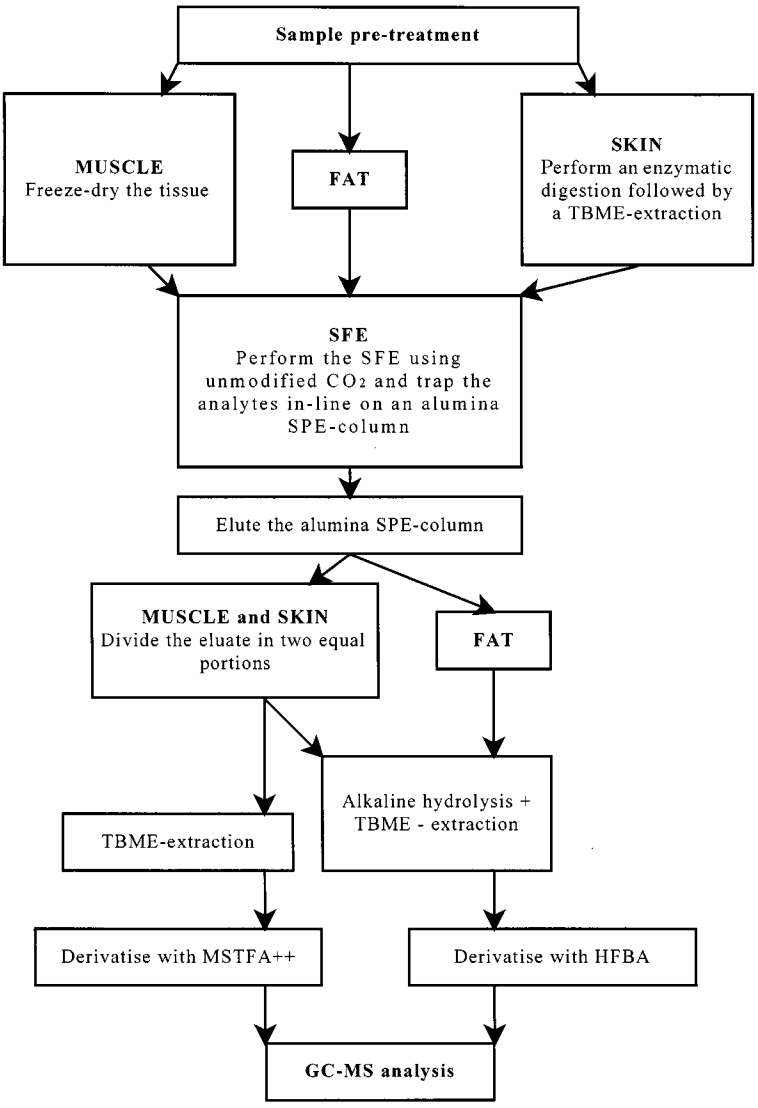

Fig. 2 Flow diagram of the method of analysis (the procedure is described in detail in in-house SOP RIVM ARO/435).

Skin. From the laboratory sample, a test portion of 5.0 g was weighed into a 50 ml glass centrifuge tube. Internal standard solution (25 μl of 1.0 ng μl^{-1}) standard solution was added and mixed with the test portion at least 30 min prior to the addition of 20 ml of 0.1 mol l^{-1} TRIS buffer, pH 9.5, containing 5 mg of subtilisin A. This enzymatic digestion was to free the analytes from cells and from conjugates with proteins (non-covalently protein bound residues). The mixture was shaken several times and incubated for 2 h at 55 °C. After the enzymatic digestion, the sample was cooled to room temperature and extracted twice with 20 ml of TBME, then the TBME was evaporated. The dry residue was dissolved in 3 ml of TBME. The TBME extract was poured on to 3.5 g of Extrelut sorbent in a glass beaker (50 ml), the tube was washed with a second portion of 3 ml of TBME and the TBME was poured on to the Extrelut mixture. After 30 min, the TBME was evaporated and 1 ml of water was added.

Fat. The sample of fat was homogenised by cutting the sample in to small cubes, then 2 g of the sample of fat were blended with 3.5 g of Extrelut sorbent and the internal standard solution (10 μl of 1 ng μl^{-1} standard solution) and 1 ml of water were added.

SFE. For packing the SFE vessels, the outlet of the high pressure extraction vessel was sealed. The end flanges of a standard commercial 3 ml SPE column were removed so that it fitted into the extraction vessel (24 ml). The column then was packed with 2.0 g of neutral aluminium oxide and inserted into the appropriately sized Teflon sleeve (see Fig. 1). The column–sleeve assembly was inserted into the extraction vessel and seated in the sealed end. Next, a plug of polypropylene wool was tamped into the vessel on top of the SPE column–sleeve assembly. The sample mixture was poured into the extraction vessel and tightly compressed. The remaining void in the column was filled with a second plug of polypropylene wool and firmly tamped. The vessel was sealed with the end-cap. SFE was performed using the conditions described in Table 2. After the SFE, the extraction vessels were removed from the SFE instrument, unsealed and the in-line SPE column–sleeve assembly was removed from the vessel. The SPE column was removed from the Teflon sleeve and the steroids were eluted from the aluminium oxide with 6 ml of methanol–water (65 + 35; v/v).

Table 1 Standards

Analyte	M_r	Analyte	M_r
Methylboldenone	300.4	Medroxyprogesterone acetate-d_3	389.3
Methylboldenone-d_3	303.4		
Chloromadionone acetate	404.9	Megestrol acetate	384.4
Chloromadionone acetate-[^{37}Cl]	406.9	Megestrol acetate-d_3	387.4
		Melengestrol acetate	398.2
Chlorotestosterone acetate	364.2	Melengestrol acetate-d_3	401.2
Chlorotestosterone acetate-d_3	367.2	Norethandrolone	302.0
Delmadinone	358.1	Norgestrel	312.4
Ethynylestradiol	296.4	Nortestosterone	274.4
Ethynylestradiol-d_4	300.4	Nortestosterone-d_3	277.4
β-estradiol	272.2	β-Testosterone	288.4
β-estradiol-d_3	275.2	β-Testosterone-d_2	290.4
Medroxyprogesterone acetate	386.3		

Analysis of the primary extract (see Fig. 2)

Muscle/skin. The extract obtained after SFE was divided in to two equal portions. The solvent of portion 1 was evaporated and the residue was dissolved in 0.2 ml of alkaline hydrolysis solution. This mixture was incubated at 37 °C for 30 min. The hydrolysis was ended by the addition of 1.0 ml of acidic buffer. The mixture was extracted twice with 6 ml of TBME and the combined extract was evaporated to dryness under a stream of nitrogen. The residue was transferred to a derivatisation vial with 0.5 ml of ethanol. The ethanol was evaporated and 0.05 ml of HFBA–acetone (1 + 4 v/v) was added. The vial was vortex

Table 2 SFE conditions

Parameter	Static extraction	Dynamic extraction
Time/min	10	10
Oven temperature/°C	50	50
Micrometering temperature/°C	110	110
Pressure/bar (MPa)	650 (65)	450 (45)
Flow rate/l min^{-1} expanded gas	0	2

Table 3 GC-MSD conditions

Parameter	HFBA derivatives	MSTFA++ derivatives
Column	CP-SIL 5CB	CP-SIL 5CB
Injection volume/μl	5 (splitless)	5 (splitless)
Injector temperature/°C	250	250
Initial oven temperature/°C	75 (1 min)	75 (1 min)
Temperature ramp/°C min^{-1}	30	30
Final temperature/°C	210 (0.5 min)	250 (1.17 min)
Temperature of transfer line/°C	280	280
Solvent delay/min	8.35	8.5
Dwell time per ion/ms	40	40
Ions monitored	*m/z* 479 Medroxy-progesterone	*m/z* 418 Nortestosterone
	m/z 482 Medroxy-progesterone-d_3	*m/z* 421 Nortestosterone-d_3
	m/z 477 Megesterol	*m/z* 444 Methylboldenone
	m/z 480 Megesterol-d_3	*m/z* 447 Methylboldenone-d_3
	m/z 447 Melengestrol	*m/z* 446 Norethandrolone
	m/z 450 Melengestrol-d_3	*m/z* 456 Norgestrel
	m/z 497 Chloromadinone	*m/z* 436 Chlorotestosterone acetate
	m/z 499 [^{37}Cl]Chloromadinone	*m/z* 439 Chlorotestosterone acetate-d_3
	m/z 495 Delmadinone	
	m/z 664 β-Estradiol	
	m/z 667 β-Estradiol-d_3	
	m/z 474 Ethynylestradiol	
	m/z 478 Ethynylestradiol-d_4	
	m/z 320 Testosterone	
	m/z 322 Testosterone-d_2	

mixed and incubated for 1 h at 60 °C. After incubation, the reaction mixture was evaporated to dryness under a stream of nitrogen at 50 °C and the derivatised residue was dissolved in 0.025 ml of isooctane and transferred into a GC injection vial with a micro-insert.

The solvent of portion 2 was evaporated under a stream of nitrogen in a water-bath at 50 °C, 2 ml of water were added and the mixture was vortex mixed for 30 s. The mixture was extracted twice with 6 ml of TBME. The TBME fractions were collected, the TMBE was evaporated and the residue was transferred into a derivatisation vial with 0.5 ml of ethanol. The ethanol was evaporated and 25 μl of MSTFA–ammonium iodide–dithioerythritol (1000 + 2 + 4, v/w/w) (= MSTFA++, Fig. 2) were added. The vial was mixed for 30 s and incubated for 1 h at 60°C. After incubation, the reaction mixture was evaporated to dryness under a stream of nitrogen at 50 °C and the derivatised residue was dissolved in 0.025 ml of isooctane and transferred into a GC injection vial with a micro-insert.

Fat. The extract obtained after SFE of the sample of fat was hydrolysed and derivatised in the same way as described for portion 1 of the samples of muscle/skin.

GC-MS analysis. The samples and standard solutions were analysed by GC-MS using the conditions given in Table 3 and the single ion monitoring (SIM) mode. Standards were derivatised corresponding to a mass concentration range from 0 to 10 μg kg^{-1}.

In case of a positive screening result, the derivatised extract was re-injected and analysed for the presence of more ions diagnostic for the compound detected.

Interpretation and calculation

The areas of the relevant selected ion chromatograms obtained for the standards and internal standards (IS) were integrated to provide quotients of response. A calibration curve was constructed by analysing different concentrations of standard. A linear curve was fitted using a least squares linear regression calculation. Unknown concentrations were calculated by interpolation.

For identification according to the EC criteria,[7] it is mandatory that at least four ions are monitored. The minimum acceptable response for each of the ions monitored should fulfil the criterion that the individual signal peak maxima (*S*) should exceed the average channel noise level by at least three standard deviations (*R*). If this criterion is fulfilled, the three different ratios were calculated. The same ratios were calculated for the standard analyte, preferably at a corresponding concentration. For positive identification, the responses obtained for the unknown sample should preferably all be within ±10% of the average value for the standard.

Method validation

The limit of detection is defined as the analyte concentration at which *S/R* = 3 was obtained for the most intense ion monitored. The limit of identification was defined as the analyte concentration at which the response for the least intense ion, of the four ions selected, provided an *S/R* of 3.

The repeatability and reproducibility of the analyte quantification were checked by analysing fortified samples on three days in triplicate. From the samples analysed the peak areas of

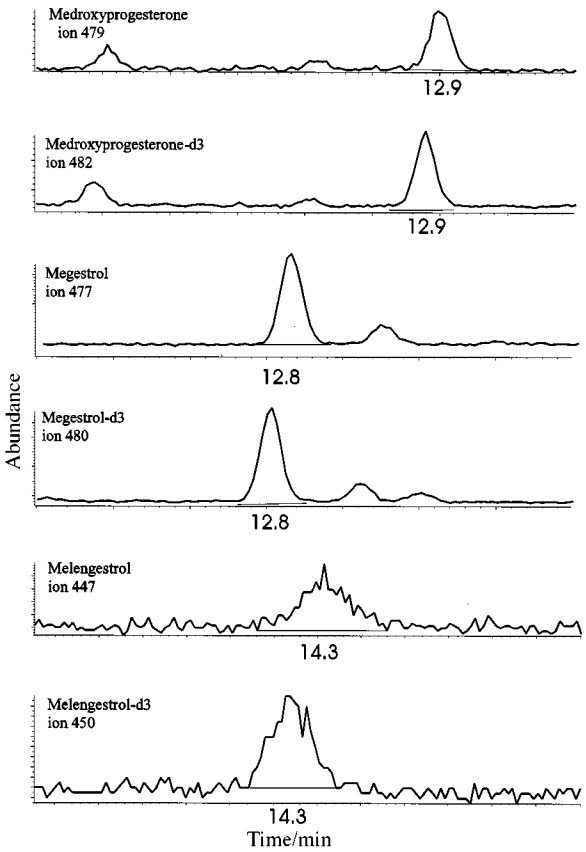

Fig. 3 GC-SIM-MS ion chromatograms of HFB derivatives of medroxyprogestrone, megestrol and melengestrol and the corresponding deuterated compounds isolated from fortified (5 μg kg^{-1}) samples of bovine kidney fat by using the SFE-SPE technique.

Table 4 Field of application

Analyte	Bovine muscle	Bovine skin	Bovine (kidney) fat	Limit of detection/μg kg^{-1}	Limit of identification/μg kg^{-1}
Methylboldenone	×[a]	×		2	2–5
Chloromadionone acetate	×		×	2	2–5
Chlorotestosterone acetate	×	×		2	5
Delmadinone	×		×	2	5
Ethynylestradiol	×	×		2	2–5
β-Estradiol	×	×		2	2–5
Medroxyprogesterone acetate	×		×	2	2–5
Megestrol acetate	×		×	2	2–5
Melengestrol acetate	×		×	5	5
Norethandrolone	×	×		2	2–5
Norgestrel	×	×		2	2–5
β-Nortestosterone	×	×		2	2–5
β-Testosterone		×		2	2–5

[a] Combination of analyte–matrix which can be analysed by the described SFE method.

analyte and internal standard were measured and the analyte/IS ratio was calculated. The mean analyte/IS ratio for every analyte–matrix combination was calculated within day and between days. The repeatability was expressed as the RSD of the mean ratios within day and the (within-laboratory) reproducibility was expressed as the RSD of the mean ratios between the three individual days.

Results

The method described was applied to different analyte–matrix combinations. Table 4 shows the different combinations and their limits of detection and identification. Fig. 3–5 show the different chromatograms obtained for the different matrices, fat, muscle and meat. Tables 5–7 give the validation results of the method. Currently not all analyte–matrix combinations have been validated.

Discussion

A limitation of SFE is the size of the sample that can be used for extraction. For the samples of muscle tissue this problem could be solved by freeze-drying the sample. Freeze-drying 5 g of wet animal tissue results in approximately 1.5 g of dry material. Starting with a sample of 5 g, instead of 2 g, which is the maximum for direct extraction, lowers the limit of detection from 5 to 2 μg kg^{-1}. For the samples of skin, freeze-drying was not possible. This tissue has a very low moisture percentage so freeze-drying did not significantly reduce the mass of the sample. Another problem with the samples of skin was the compactness of the sample. To obtain a reproducible CO_2 flow through the sample, a homogeneous mixture of sorbent (*e.g.* Extrelut Celite, Hydromatix) and tissue must be obtained. The samples of skin were too leathery and mixing with a sorbent was not possible. By performing an enzymatic digestion with protease followed by a quick TBME extraction, the final residue, obtained after evaporation of the TBME, could be used for SFE. For the samples of fat, at present the amount of sample which can be used is still the limiting factor for decreasing the limit of detection.

In this study, in-line collection of analytes (trapping the analytes under supercritical conditions) was necessitated by the

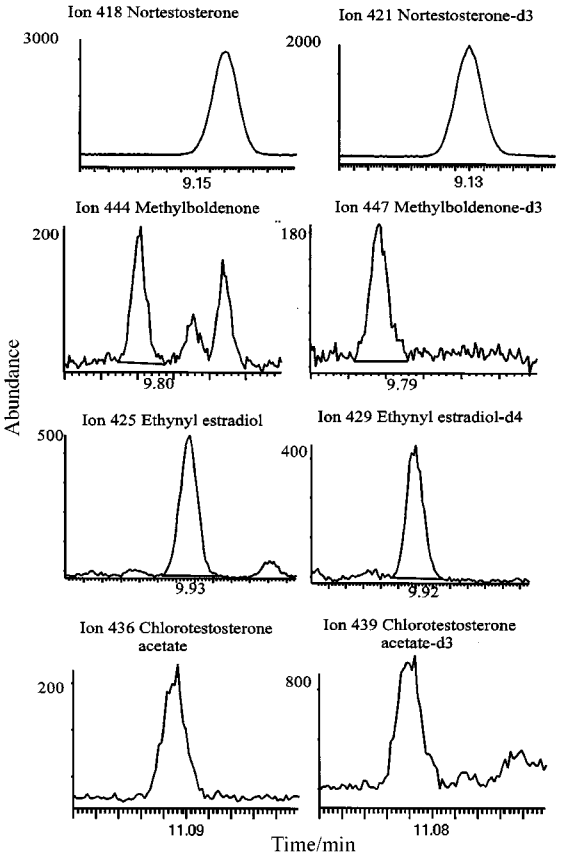

Fig. 4 GC-SIM-MS ion chromatograms of TMS derivatives of steroids and the deuterated internal standards, isolated from fortified (5 μg kg^{-1}) samples of bovine muscle.

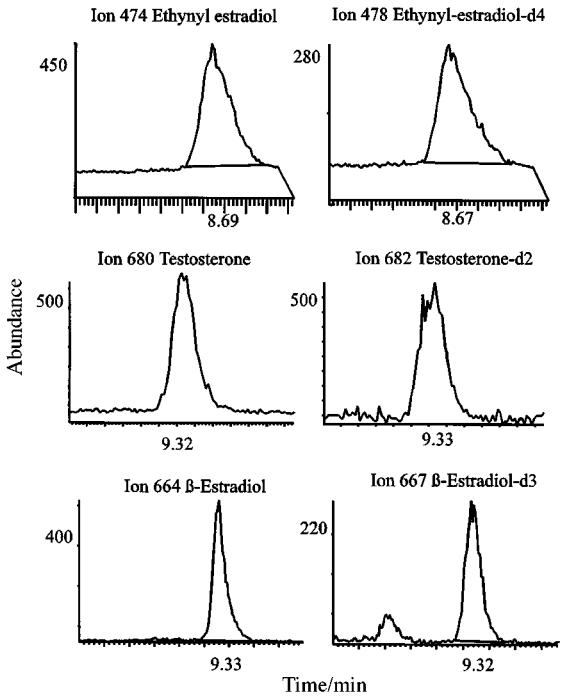

Fig. 5 GC-SIM-MS ion chromatograms of HFB derivatives of steroids and the deuterated internal standards, isolated from fortified (5 μg kg^{-1}) samples of bovine skin.

Table 5 Validation results of SFE-GC-MS of gestagens from fortified bovine kidney fat

Analyte[a]	Repeatability, RSD (%) (n = 9)	Within-laboratory reproducibility, RSD (%) (n = 3)
Medroxyprogesterone acetate	13	39
Melengestrol acetate	14	13
Chloromadinone acetate	5	7
Megestrol acetate	8	20
Delmadinone acetate	11	84[b]

[a] Fortification level 5 μg kg^{-1}. [b] No deuterated internal standard was available so only qualitative analysis was possible.

Table 6 Validation results of SFE-GC-MS of steroids from fortified bovine muscle

Analyte[a]	Repeatability, RSD (%) (n = 9)	Within-laboratory reproducibility, RSD (%) (n = 3)
Methylboldenone	12	23
β-Nortestosterone	4	2
Ethynylestradiol	7	9
Chlorotestosterone acetate	5 (n = 6)	4
Norethandrolone	21	37[b]
Norgestrel	15	31[b]

[a] Fortification level 5 μg kg^{-1}. [b] No deuterated internal standard was available so only qualitative analysis is possible.

nature of the sample matrix. SFE conditions determined to be ideal for the extraction of steroids also resulted in the extraction of 1 g of fat per 2.0 g of kidney fat. Without adsorptive extraction of the steroids from the co-extracted fat in the supercritical CO_2, little would be gained by the SFE process. The addition of H_2O to the tissue–Extrelut mixture improved the procedure in several ways. It increased the absolute recoveries and reduced the background levels during final GC-MS analysis, probably owing to the disruption of analyte–matrix retentive interactions and changed polarity of the extraction solvent.

The validation results in Tables 5–7 show that good repeatability and reproducibility are obtained for the less polar steroids such as chloromadinone acetate, megestrol acetate, chlorotestosterone acetate, nortestosterone, testosterone and ethynylestradiol. The more polar steroids such as methylboldenone and estradiol are more difficult to extract reproducibly.

Some of the RSDs for the repeatability and the within-laboratory reproducibility are still relatively high, which can be partly explained by the fact that the SFE system used is a manual system. For every experiment the gas flow and other parameters are adjusted manually. The use of an automated system would possibly have a positive influence on the validation results. However, when using one method for different combinations of analyte and matrix, compromises always must be made. Whenever a sample of tissue contains residues of illegal steroids it is preferable to perform a confirmatory analysis with a method optimised for the specific analyte–matrix combination concerned.

Table 7 Validation results of SFE-GC-MS of steroids from fortified bovine skin

Analyte[a]	Repeatability, RSD (%) (n = 9)	Within-laboratory reproducibility, RSD (%) (n = 3)
β-Estradiol	40	27
Ethynylestradiol	11	23 (n = 2)
β-Testosterone	12 (n = 6)	2 (n = 2)
β-Nortestosterone	6	14
Methylboldenone	42 (n = 6)	32
Chlorotestosterone acetate	10	18

[a] Fortification level 5 μg kg^{-1}.

Conclusions

With the described method, based on SFE-SPE and GC-MS, a multi-analyte, multi-matrix method has been developed for the determination of steroids in samples of animal tissue. Depending on the analyte–matrix combination and the availability of a deuterated internal standard, quantitative or qualitative results can be obtained. Identification limits of 2–5 μg kg^{-1} are possible and even though not all the analyte–matrix combinations were completely validated, the method shows acceptable repeatability and reproducibility.

By using the SFE-SPE method, the time consuming and laborious steps of LLE and HPLC fractionation can be reduced. A sample throughput of eight samples per day is possible. The limitation on the number of samples which can be analysed is the use of the manual SFE system. The next modification of the method will be the introduction of an automated SFE system.

References

1 CEC, *Veterinary Drug Residues. Residues in Food Producing Animals and Their Products: Reference Materials and Methods*, ed. R. J. Heitzman, Report EUR 15127. Blackwell, Oxford, 1994.
2 A. A. M. Stolker, M. A. Sipoli Marques, P. W. Zoontjes, L. A. van Ginkel and R. J. Maxwell, *Semin. Food Anal.*, 1996, **1**(2), 101.
3 N. Din, K. D. Bartle, and A. A. Clifford, *J. High Resolut. Chromatogr.*, 1996, **19**, 465.
4 R. P. Huopalahti and J. D. Henion, *J. Liq. Chromatogr. Relat. Technol.*, 1996, **19**, 69.
5 R. J. Maxwell, A. R. Lightfield and A. A. M. Stolker, *J. High Resolut. Chromatogr.*, 1995, **18**, 231.
6 O. W. Parks, R. J. Shadwell, A. R. Lightfield and R. J. Maxwell, *J. Chromatogr. Sci.*, 1996, **34**, 353.
7 Commission Decision 93/256/EEC, *Off. J. Eur. Commun.*, **1993,** L-118/64.

Paper 8/05127E