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NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

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Multi residue method using coupled-column HPLC and GC-MS for the determination of anabolic compounds in samples of urine.

H.A. Herbold, S.S. Sterk, R.W. Stephany and L.A. v. Ginkel.

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29

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H.A.Herbold, S.S. Sterk, R.W. Stephany en L.A. v. Ginkel.

Samenvatting

Dit rapport beschrijft een multiresidu methode voor de analyse, detectie en identificatie van residuen van anabolica. De analytische methode is gebaseerd op een geautomatiseerde vaste fase-extractie en een High Performance Liquid Chromatography (HPLC) kolomschakelingssysteem. Detectie vindt plaats met Gas Chromatografie met Massa Spectroscopische Detectie (GC-MS). De methode is geschikt voor de screening van urine monsters op 17 anabolica en de metabolieten daarvan. Gedeutereerde analoga worden gebruikt als interne standaarden voor kwantificering en kwaliteitscontrole.
Na screening kan de identiteit van de anabolica bevestigd worden door het gebruik van een gemodificeerde HPLC-procedure met gradiëntelutie en selectieve fractionering.
De geautomatiseerde extractie wordt uitgevoerd door een Gilson ASPEC® systeem met wegwerp vaste fase extractie kolommen gekoppeld aan een HPLC met kolomschakeling. Het kolom schakelsysteem bestaat uit twee analytische kolommen gekoppeld via een schakelklep. Na extractie en HPLC zuivering wordt voor de screening één enkele fractie uitgevangen die alle analieten bevat. Deze fractie wordt in tweeën gedeeld en gederivatiseerd met twee verschillende derivatiseringsreagentia voor analyse met GC-MS.
De detectielimiet voor alle componenten, behalve diethylstilbestrol (DES), is 1 µg/l. Voor DES bedraagt deze limiet 0.1 µg/l. De meerderheid van de componenten heeft een nauwkeurigheid tussen de 90 en 110 %. Voor drie componenten (4-chloro-4-androst-3,17-dione, methyltestosterone en 17-ß-nortestosterone) werd een grotere spreiding waargenomen. Voor alle componenten is de nauwkeurigheid acceptabel.
Deze screeningsmethode is getest op vals negatieve resultaten door het analyseren van runder- (n=20), schapen- (n=20) en varkens- (n=10) urines op het concentratie niveau van één en twee maal de detectielimiet. De resultaten werden acceptabel geacht als het aantal vals negatieve resultaten kleiner of gelijk was aan 5%. Voor runder- en varkensurines op het niveau van 1 µg/l werd aan deze eis voldaan. Voor monsters schapenurines werden de resultaten op het concentratie van 2 µg/l geaccepteerd.
De methode kan ook gebruikt worden als bevestigingsmethode. Voor dit doel wordt de te bevestigen analiet selectief geïsoleerd door HPLC met gradiëntelutie en fractionering. De uitgevangen fractie wordt gederivatiseerd en bevestigd met GC-MS door het meten van ten minste 4 specifieke massafragmenten.
De analysemethode is in detail beschreven in ARO SOP 401.

NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

Multi residue method using coupled-column HPLC and GC-MS for the determination of anabolic compounds in samples of urine.

H.A.Herbold, S.S. Sterk, R.W. Stephany and L.A. v. Ginkel.

Summary

This report describes a multi-residue method for the detection and identification of residues of anabolic compounds. The method is based on automated sample preparation (Solid Phase Extraction (SPE)) combined with coupled-column High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry (GC-MS). The method is suitable for the screening of samples of urine for the presence of 17 anabolic compounds or their metabolites. For purposes of quantification and quality control (QC) a number of deuterated internal standards is used. The extraction of samples of urine is performed automatically with a Gilson Aspec® System equipped with disposable SPE columns in combination with coupled column reversed phase HPLC. The coupled-column system consists of two analytical columns coupled via a switching valve.
After extraction and HPLC clean-up a single fraction is collected containing all the analytes. This fraction is divided into 2 parts for derivatization with two different procedures and final determination with GC-MS. The limit of detection for all compounds, except diethylstilbestrol (DES), is 1 µg/l (ppb). For DES this limit is 0.1 ppb. For the majority of compounds the accuracy of the determination is between 90% and 110%. For only 3 compounds (CLAD, MT and 17β-NT) slightly larger deviations were observed. In all cases the accuracy is considered acceptable.
The procedure for screening was tested for the occurrence false negative results by spiking samples of bovine- (n=20), ovine- (n=20) and porcine urine (n=10) at the level of the limit of detection and two times this level. The results are considered "acceptable" if the number of false negative results is equal to or below 5%. The results for samples of bovine and porcine urine were acceptable at the level of 1 µg/l. For samples of ovine urine the results were acceptable at the level of 2 µg/l.
The method can be used for confirmation for which purpose the target analyte is isolated selectively by HPLC with gradient elution. The fraction obtained is suitable for confirmation of the analyte based on the detection of at least four ions.
The procedure is described in detail in SOP ARO/401.

1. Multi-residue Method for screening

1.1 Introduction

One of the tasks of the EU Community Reference Laboratories (CRL) for residues is to provide the National Reference Laboratories (NRLs) with adequate methods of analysis to be used within the Annual National Control Programmes (ANPs). Within the area of (illegal) hormonal growth promoting compounds (anabolics) this task is complicated due to the wide variety of, ever changing, analytes for which control is necessary. Samples have to be analysed for a, regularly updated, set of compounds for which purpose flexible multi-residue methods are necessary (1-3,6).
For the purpose of regulatory and surveillance analyses frequently a two step model is used. During the first step all samples are analysed with a, relative simple, method to discriminate between "negative" samples and "not-negative" samples. Samples that are "not-negative" are subsequently analysed with a second, usually more sophisticated, method. The methods used during screening are developed in such a way that the number of false negative results is minimized whereas the criterion for false positive results is less essential since all "not-negative" samples are re-analyzed. The method used in this second confirmative step is developed in order to prevent false positive results by using more sophisticated detection principles, yielding more direct information on the structure of the compound detected. This report describes the development and validation of two methods, both based on Gas Chromatography-Mass Spectrometry (GC-MS) for the purposes of respectively screening and confirmation. The objectives set for the methods described in this report were:

- the method had to be suitable for screening for a large number of anabolic compounds.
- the number of false negative results at the level of 1 ppb had to be equal to or below 5 %.
- the limit of determination had to be equal to or below 2 ppb.
- the method had to be automated, allowing a high sample throughput.
- the method had to be flexible, allowing the quick incorporation of new compounds.

The study was started with the development of the method for screening. After developing and (partly) validating this method research was continued in order to optimize the method for confirmation (identification) under the conditions layed down in European legislation (4).

A variety of methods for screening exists for the determination of anabolic compounds in biological samples. Methods were reported using only HPLC column-switching techniques as well as methods including GC-MS detection, after a variety of derivatization techniques. The limitations of many of these methods is the limited number of compounds that can be detected as well as the low sample throughput. In this report we describe an automated multi-residue method for analysing 17 anabolic compounds simultaneously with a sample throughput of 14 samples a day. Following the description of the method for screening, modifications of this procedure are described for confirmatory analysis.

1.2 Materials and method

More details of the procedures used are described in SOP 401 (9). In this chapter the method used for screening is described. Figure 1 gives a schematic overview of the equipment used during clean-up.

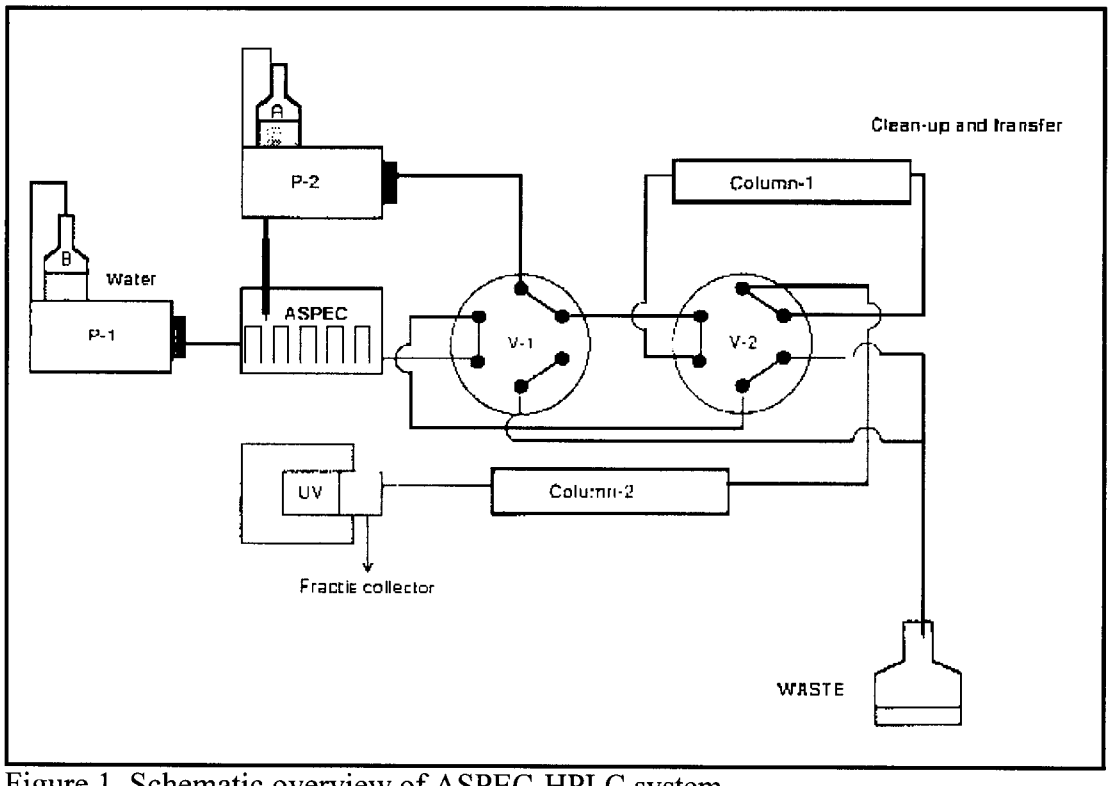


Figure 1. Schematic overview of ASPEC-HPLC system.
Schematic setup of the coupled-column HPLC system. ASPEC® = Automated sample preparation with extraction columns; V-1 and V-2 = valves; P-1 and P-2 = LC pumps; UV = UV-vis detector, A = methanol/water (70:30), B = water.

1.2.1 Reagents[1]

SPE extraction column: 6 ml disposable octadecyl (C18)
SPE extraction column: 6 ml disposable amino (NH2)
Two HPLC columns LiChrospher 100 EcoCart 125-3 RP-18 (5 mm)(Merck)
Methanol (Baker Analyzed nr.8045)
Ethanol (Baker Analyzed, nr.8006)
Acetone (Baker Analyzed, nr.8001)
Iso-octane (Baker Analyzed nr.8715)
Acetone (Romil, high dry, anhydrous solvent nr.E450433.
Heptafluorobutyric Acid Anhydride (Pierce, nr.63164)
N-methyl-N-trimethylsilyltrifluoroacetamide(MSTFA)(Macherey-Nagel, nr.70127)
Ammonium iodide (Fluka, nr.09874)
Dithioerythriol (Sigma, nr.D-9779)
Iso-octane (Merck, art. no.4718)

[1] Reference to a product or company is for purposes of identification and information only and does not imply any recommendation by the CRL or RIVM of the company.

Acetic acid (Merck, art. no.63)
Sodiumacetate (Merck, art. no. 6268)
Beta-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100.000 units ß-glucuronidase and 100.000 units sulfatase per ml, France, code IBR 213473).
Acetate buffer, 2 mol/l, pH 5.2. (25.2 g of acetic acid and 129.5 g sodiumacetate in 800 ml of water, pH 5.2±0.1, final volume 1000 ml.
HPLC solvent: methanol:water (7:3)(v/v).
N, O, -bis(trimethylsilyl)trifluoroacetamide (CAS 25561-30-2) with 1% trimethylchlorosilane (CAS 75-77-4). (Pierce, No.38831)

1.2.2 Internal Standards

Internal standards used are listed in Table 1.
From the internal standards stock solutions containing 1 mg/ml are prepared. The stock solutions are prepared by dissolving the appropriate amount of the analytes in ethanol. Quality Control includes the registration of a mass spectrum (confirmation of identity).
The solutions are stored in the dark at approximately -20°C for a maximum period of 5 years. Working solutions are prepared by 10-fold dilutions of the stock solutions with methanol. These solutions are stored in the dark at 4°C (range 1-10°C) for a maximum period of 6 months.

Table 1: Isotope enriched internal standards.

Internal standard	Abb.	RIVM/ARO nr.	Source
diethylstilbestrol-d6	Des-d6	H145661	RIVM
zeranol-d4*	Zer-d4	87M1553	RIVM
taleranol-d4*	Tal-d4	87M1553	RIVM
α-zearalenol-d4	α-Zer-d4	95M1936	RIVM
ß-zearalenol-d4	b-Zer-d4	95M1937	RIVM
17ß-19-nortestosterone-d3	17ß-NT-d3	87M1056	RIVM
17ß-Trenbolone-d2	17ß-Tb-d2	95M1559	RIVM
17ß-estradiol-d3	17ß-E2-d3	89M1691	MSD md-2325
3-chlortestosterone-d3	3 Chl.T.-d3	94M0585	Liège (B)
1-dehydromethyltestosterone-d3	MBol-d3	95M0465	RIVM
17ß-testosterone-d2	17ß-T-d2	89M1692	MSD MD-2962
17α-ethynyl estradiol-d4	17α-EE2-d4	94M5558	C/D/N isotopes D4319

*Zeranol-d4 and taleranol-d4 are a mixture of ± 50% of each compound.

1.2.3 Standards

Standards used are listed in Table 2.
From these standards stock solutions containing 1 mg/ml are prepared. The stock solutions are prepared by dissolving the appropriate amount of the analytes in ethanol. Quality Control includes the registration of a mass spectrum (confirmation of identity).
These solutions are stored in the dark at approximately -20°C for a maximum period of 5 years. Working solutions are prepared by 10-fold dilutions of the stock solutions with methanol. These solutions are stored in the dark at 4°C (range 1-10°C) for a maximum period of 6 months.

Table 2. Analytes included in the method.

analyte	abr.[2]	CAS #	formula	Mwt
17α-19-nortestosterone	17α-NT	4409-34-1	$C_{18}H_{26}O_2$	274.3
17ß-19-nortestosterone	17ß-NT	434-22-0	$C_{18}H_{26}O_2$	274.3
17α-testosterone	17α-T	481-30-1	$C_{19}H_{28}O_2$	288.4
17ß-testosterone	17ß-T	58-22-0	$C_{19}H_{28}O_2$	288.4
17α -methyltestosterone	MT	58-18-4	$C_{20}H_{30}O_2$	302.4
17α -1-dehydrotestosterone	α-Bol	RIVM	$C_{19}H_{26}O_2$	286.4
17ß-1-dehydrotestosterone	ß-Bol	846-48-0	$C_{19}H_{26}O_2$	286.4
17α -estradiol	17α-E2	57-91-0	$C_{18}H_{24}O_2$	272.2
17ß-estradiol	17ß-E2	50-28-2	$C_{18}H_{24}O_2$	272.2
17α -ethynyl-estradiol	17α-EE2	57-63-6	$C_{20}H_{24}O_2$	296.4
diëthylstilbestrol	DES	56-53-1	$C_{18}H_{20}O_2$	268.4
4-chloro-4-androst-3,17 dione	CLAD	RIVM	$C_{19}H_{27}CLO_3$	320
1-dehydromethyltestosterone	MBol	72-63-9	$C_{20}H_{28}O_2$	300.4
Zeranol	Zer	26538-44-3	$C_{18}H_{26}O_5$	322.4
taleranol	Tal	42422-68-4	$C_{18}H_{26}O_5$	322.4
α-zearalenol	α-Zer	36455-72-8	$C_{18}H_{24}O_5$	320
ß-zearalenol	ß-Zer	71030-11-0	$C_{18}H_{24}O_5$	320

1.2.4 Sample Materials

Blank samples used during method validation from the CRL "Bank of Reference Blank Samples" which contains samples of lyophilised bovine-, ovine- and porcine urine of known origin.

1.2.5 Equipment

ASPEC® (Automated Sample Preparation with Disposable Extraction Columns) XL Gilson, (Meyvis, Bergen op Zoom, the Netherlands)
The ASPEC® consists of three components, a model 401C dilutor, a sample processor and a set of racks and accessoires to handle 1 ml and 6 ml SPE columns. The system is equipped with an injection-valve (loop-volume 10 ml) for on-line injection of the last eluate into the HPLC system. The ASPEC® is programmed using the Gilson Sample Manager 721 software. A "MUST" -system (MUST-IET:Multi-port Streamswitch, Spark Separation: Hendrik Ido Ambacht, The Netherlands) couples two analytical columns to the ASPEC® system and the HPLC -system (Fig.1).

2= abbreviation used in this report.

HPLC-system (Pharmacia LKB; Woerden, The Netherlands)
The HPLC-system consists of two LKB 2150 HPLC-pumps, a LC-Controller and a Spectra Focus absorbance detector (UV).
Collecting tubes for the ASPEC® system, Gilson type B54728-4.
Spectra Focus absorbance detector (UV)(Spectra Physics).
Derivatization vials, Screw Top Vial with Silicone/PTFE Septa (OMNILABO, Cat.No.154920).
Automatic pipettes (Gilson P20, P100, P200, P1000 and P5000)
Injection vials, Wide Mouth Crimp (Alltech, nr.98213), with micro inserts (200 ml) (Alltech EK-1022.395).
Vortex (Vortex-genie, Wilton & Co).
pH-meter (Applikon).
Fraction collector (Pharmacia), type Frac200.

1.2.6 GC-MS equipment

The chromatographic analysis of the extracts is performed on a Hewlett Packard 5890 serie 11 gaschromatograph equipped with a Hewlett Packard 7673 automatic sampler, a Hewlett Packard Vectra computer 486/66U with Hpchem data acquisition software and a 5989A Mass Spectrometer (Engine).
Fused silica capillary column CP SIL-5 (Low bleeding/non polar). Chrompack (Cat.no.7818), length 60 meter, i.d. 0.25mm, 0.1 micron film thickness.

1.3 Analytical procedure for screening

1.3.1 Sample pretreatment

Urine (10 ml) is spiked with a mixture of internal standards (Table 1) and 20 µl Suc Helix Pomatia. The pH is adjusted to 5.2 and 2 ml of 2 mol/liter acetate buffer is added. The mixture is vortexed for 30 seconds and hydrolysed over night at approximately 37 °C. After hydrolysis the mixture is centrifuged for 2 minutes at 3600 rpm.

1.3.2 Solid Phase Extraction with ASPEC®

The ASPEC® system is equipped with 5 racks on the tray platform (Figure 2). There is a solvent-rack for 4 different solvents, a sample-rack for 14 sample tubes, 3 racks for SPE columns and special racks for the collection tubes.

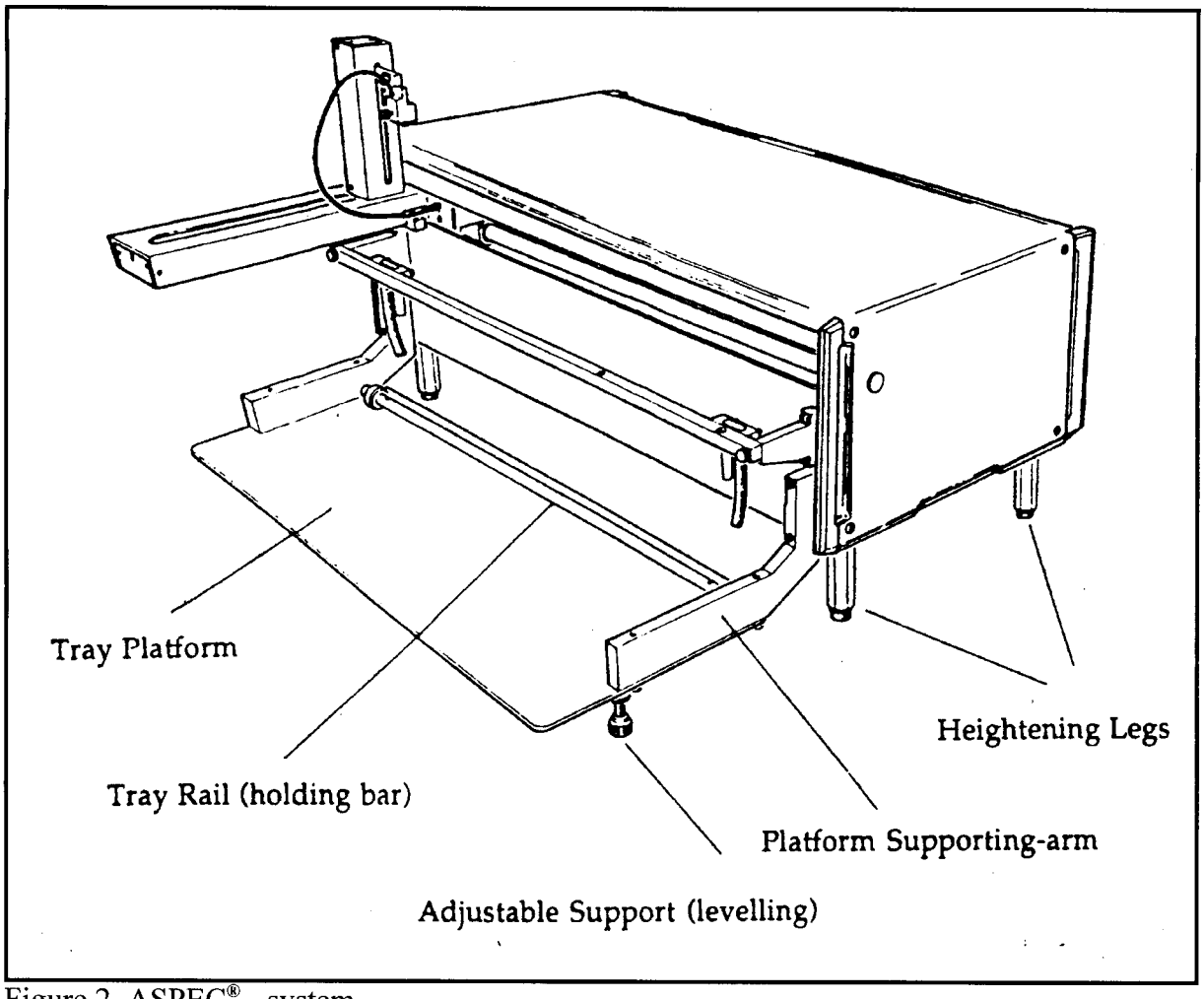


Figure 2. ASPEC® -system.

The pretreated samples are placed in the sample-rack on the tray platform. In the solvent rack methanol, methanol:water (40:60) and acetone are placed. The 6 ml C18 (SPE) disposable columns and 6 ml amino NH2 columns are fitted with polypropylene caps and installed in the SPE column racks. The collection tubes are placed in the collection racks.
The system is programmed using the Gilson Sample Manager 721 software. The ASPEC® system can be programmed directly from the Gilson control panel.

A listing of the program used is given in RIVM ARO SOP 401. All programs are stored and operated from a 3.5" disk.

The extractions are performed automatically according to the steps decribed below:

1. The SPE column racks are reset to the extraction position by the needle of the 401 dilutor (Figure 3a).
2. The (6 ml) C18 column is preconditioned by passing 5 ml of methanol and 5 ml of milli-Q water through the column at a flow rate of 6 ml/min.
3. Of the pretreated sample 9.9 ml is aspirated from the sample tube and dispensed onto the C18 column. The sample is passed through the column by pressure at a flow rate of 6 ml/min. Air (2 ml) is passed through the column to push residual sample through.
4. The C18 column is washed with 5 ml of milli-Q water and a mixture of 4 ml of methanol/milli-Q water (40:60) at a flow rate of 6 ml/min. Air (2 ml) is passed through the column to remove residual wash solvent. The SPE column rack is moved to the collection position.
5. The eluens (3 ml of acetone) is pushed through the column at a flow rate of 6 ml/min and the eluate is collected in a tube. Air (2 ml) is passed through the column to remove the eluens completely.
6. The amino column is preconditioned by passing 5 ml of methanol/milli-Q water (60:40) through the column at a flow rate of 6 ml/min. Subsequently, the SPE column rack is moved to the collection position.
7. The collected eluate (3 ml of acetone) is aspirated from the collection tube and dispensed onto the amino column. The eluate is passed through the column by pressure at a flow rate of 6 ml/min. Air (2 ml) is passed through the column to remove residual solvent. The eluate of this extraction step is collected in a collection tube, diluted with 7.0 ml of water and mixed.
8. The total eluate (10 ml) is injected onto column-1 of the HPLC system (flow rate 0.6 ml/min). For the first 20 minutes following injection, column-1 effluent is sent directly to the waste. (The eluens is water to remove the acetone). Column-1 is coupled, via a switching valve, to column-2 and analytes elute with a mixture of methanol water (70:30) from column-2, at a flow rate of 0.6 ml/min. A part of the eluate is collected with a fraction collector.
9. The first 4 minutes after column-switching (8) effluent is sent to the waste to prevent it from interfering with early eluting analytes. After 4 minutes, a fraction of 15 minutes (9 ml) is collected.

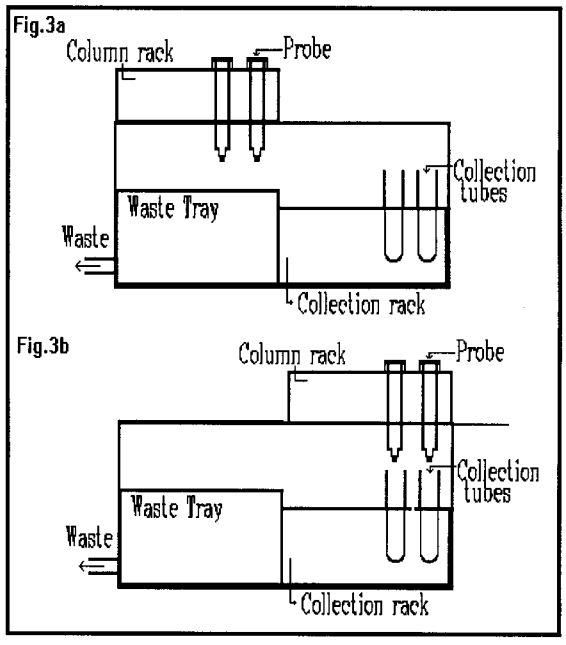


Figure 3 (a= extraction- b= collection-position)

- The time required for trapping the analytes on the first column is the injection time. The injection time depends on the volume injected and the flow rate of the HPLC pump. In this case the injection time must be at least 20 minutes because the flow of the HPLC pump is 0.6 ml per minute, the volume injected is 10 ml and the tubing between injection valve and "MUST"-system has a volume of approximately 2 ml (Figure 1).

- The moment the fraction collector starts collecting the eluate is the moment at which the separation between the interfering compounds originating from the sample and the first analyte is optimal (Figure 4).

The ASPEC® continues with the extraction of the next sample until all the samples are processed.

1.3.3 Derivatization

The eluates are evaporated at 50 °C under a stream of nitrogen until dryness and dissolved in methanol (0.5 ml). The extract is separated into two parts of approximately 250 ml and transferred into two different derivatization-vials and evaporated at 50 °C under a stream of nitrogen until dryness. One part of the dry residue is derivatized with 25 µl of Heptafluorobutyric Acid Anhydride/acetone p.a. (1:4). The residue in the other vial is derivatized with 20 µl of N-methyl-N-trimethylsilyltrifluoroacetamide(MSTFA)-ammoniumiodide-dithioerythritol(1000:2:4, v/w/w). The derivatization-vials are placed in a heater at 60 °C for 1 hour. The vials are evaporated at 50 °C under a stream of nitrogen to dryness and dissolved in iso-octane (20 µl) and transferred to injection-vials with micro inserts. The 7673 automatic sampler injects 2 µl into the GC-MS.

1.3.4 GC-MS conditions

The gas chromatograph is equipped with a 60 meter CP SIL-5 (Low Bleeding/non polar) fused silica capillary column (0.25 mm i.d., 0.1 micron film thickness, SGE). The oven temperature is held at 80 °C for 1 min, then increased with 30 °C/min. to 300 °C and held at this final temperature for 5 min. The injector temperature is maintained at 250 °C, the detector temperature at 250 °C for the MS-source and at 120 °C for the MS-quadrupool. For quantitative analysis a selected ion monitoring (SIM) program is used. For the diagnostic ions see Table 3.

The instrument is operated in the electron-impact (EI) ionization mode. The injector port is set in the splitless mode, and after 0.75 minutes in the split mode. The helium carrier gas flow rate is 0.6 ml/min. The Electronic Pressure Control (EPC) system is used for constant flow. The pressure is held at 50 psi for 1 minute, then decreased with 99 psi/minute to 28.4 psi. The flow through the system is constant during the oven-temperature program.

1.4 Quality control

1.4.1 Column-switching

It is important to check that column switching occurs at the correct moment. To check this, a standard solution of 17ß-NT[3] containing 1 µg per ml is used. This solution is injected into the injection valve of the HPLC-system, with the valve V-2 of the "Must" system (Figure 1) in the "waste" position. The mobile fase is milli-Q-water and the flow 0.6 ml per minute. After the injection time, the valve V-2 is switched to the "elute" position and both analytical columns are eluted with a mixture of methanol and water (70:30 (V:V)). The UV-detector is used at 254 nm. After approximately 4 minutes, most interfering matrix compounds are eluted (Figure 4). The switching point for changing the wait mode of the fractioncollector in the collection mode is 4 minutes after the moment of injection. This switching point has to be checked regularly.

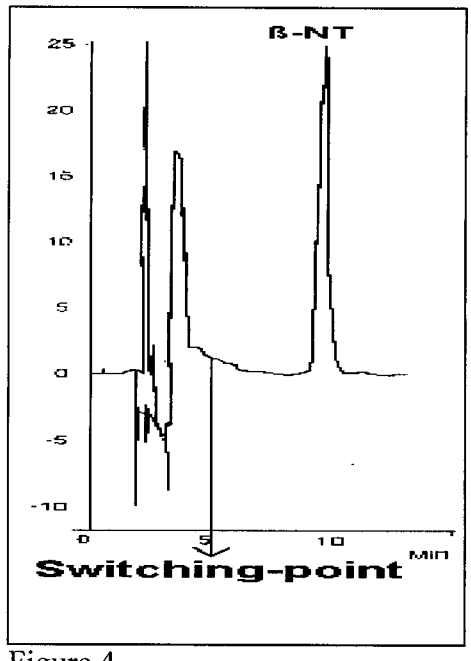


Figure 4.
Switching-point = moment of switching wait mode of the fractioncollector, in the collection mode.

1.4.2 Calibration curves

A calibration curve is constructed for each compound consisting of at least 5 mass-concentrations. Peak area ratios are calculated using the linear regression program Calwer. 17α-NT, 17ß-NT, 17α-Bol, 17ß-Bol are calculated to deuterated 17ß-NT-d3; MBol to deuterated MBol-d3; MT to deuterated MT-d3 ; DES to deuterated DES-d6; CLAD to deuterated Chl.T.-d3; 17α-T, 17ß-T to deuterated 17ß-T-d2; 17α-E2, 17ß-E2 to deuterated 17ß-E2-d3; a-Zer; ß-Zer, to deuterated α-Zer-d4 and ß-Zer-d4; Zer and Tal to a mix 50% of deuterated α-Zer-d4 and 50% deuterated ß-Zer-d4; 17α-EE2 to deuterated 17α-EE2-d4.

1.5 Results and discussion

Table 3 shows that the limits of detection for the analytes in samples of bovine- and porcine urine range from 0.1 to 1.0 ppb. For samples of ovine urine the limit of detection is 2 ppb for all compounds. The limit of detection is the minimal detectable amount based on the detection of the most abundant diagnostic ion with a response at the correct retention time and exceeding the average noise + 3 SD.

[3] β-NT is chosen because this compound is the anabolic with a shortest retention time.

Table 3. Limits of detection and diagnostic ions.

Anabolic agents	Limit (ppb).	TMS	HFB	4 Diag. ions*	Internal standard
CLAD	1	m/z 464		464, 392, 357, 221	m/z 469
DES	0.1	m/z 412		412, 397, 383, 413	m/z 418
ZER	1	m/z 433		538, 433, 335, 307	m/z 437
TAL	1	m/z 433		538, 433, 335, 307	m/z 437
α-ZER	1	m/z 446		536, 446, 333, 305	m/z 450
ß-ZER	1	m/z 446		536, 446, 333, 305	m/z 450
17α-E2	1		m/z 664	664, 451, 409, 356	m/z 667
17ß-E2	1		m/z 664	664, 451, 409, 356	m/z 667
17α-NT	1		m/z 666	666, 453, 306, 133	m/z 669
17ß-NT	1		m/z 666	666, 453, 306, 133	m/z 669
α-Bol	1		m/z 678	678, 464, 369, 169	m/z 669
ß-Bol	1		m/z 678	678, 464, 369, 169	m/z 669
M-Bol	1		m/z 478	478, 367, 435, 463	m/z 481
MT	1		m/z 465	480, 465, 369, 355	m/z 468
17α-T	1		m/z 680	680, 467, 355, 320	m/z 682
17ß-T	1		m/z 680	680, 467, 355, 320	m/z 682
17α-EE2	1		m/z 474	474, 459, 446, 353	m/z 478

* the underlined mass-fragment is used in screening and is the most abundant fragment.

1.5.1 Accuracy

The accuracy was determined for the 17 compounds included in the method by analysing spiked samples of bovine urine at 4 different concentrations. The individual results have been summarized in Table 4. For the majority of compounds the accuracy is between 90% and 110%. Only for 3 compounds (CLAD, MT and 17β-NT) slightly larger deviations were found. In all cases, however, the accuracy is considered acceptable.

Table 4. Accuracy, expressed as percent of target value

Anabolic agents	Accuracy 1 ppb	Accuracy 5 ppb	Accuracy 7.5ppb	Accuracy 10ppb	Average	SD
CLAD	58 (2.5ppb)	49	88	94 (20ppb)	72	22
DES	95	102	96	97	98	3
ZER	91	98	96	96	95	3
TAL	88	99	99	99	96	6
α-ZER	90	102	98	99	97	5
ß-ZER	90	101	96	103	98	6
17α-E2	77	81	114	114	97	20
17ß-E2	97	92	106	90	96	7
17α-NT	90	105	102	97	99	6
17ß-NT	97	92	104	104	99	6
α-Bol	75	77	104	88	86	13
ß-Bol	83	129	123	89	106	23
M-Bol	89	95	94	102	95	5
MT	124	127	120	109	120	8
17α-T	84 (0.5ppb)	88 (2.5ppb)	73 (3.75ppb)	101 (5ppb)	87	12
17ß-T	136(1.25ppb)	106(2.5ppb)	107(3.75ppb)	99 (5ppb)	112	16
17EE2	98	112	111	103	106	7

1.5.2 Reliability

For methods to be used during screening the occurrence of false negative results is one of the most important criteria. This parameter was determined for the 17 compounds by analyzing 20 samples of blank bovine urine and 10 samples of blank porcine urine, all spiked at the level of 1 ppb and 20 samples of blank ovine urine at the level of 2 ppb (Figure 5). The criterion was a number of false negative results, at the level of 1 ppb (ovine 2 ppb) that is equal or below 5 %.

Figure 5 shows that for samples of bovine urine, at the level of 1 ppb, only very few false negative results occure. For methyltestosterone, Zeranol and taleranol one sample out of 20 is false negative (5%). Only for the metabolites α- and β-zearalenol an unacceptable number of false negative results was observed. For the samples of porcine urine, at the 1 ppb level, only α-testosterone (20%) and ß-zearalenol (20%) gave higher false negative scores (since n=10, one false negative results corresponds with 10%). Because the samples of ovine urine contained more interfering compounds these were tested at the 2 ppb level. The results were not satisfactory for Zeranol and taleranol (because of interfering peaks at these retention

times) and for α - and ß- boldenone (30 and 25%). Also for α-nortestosterone the percentage of false negative results was too high.

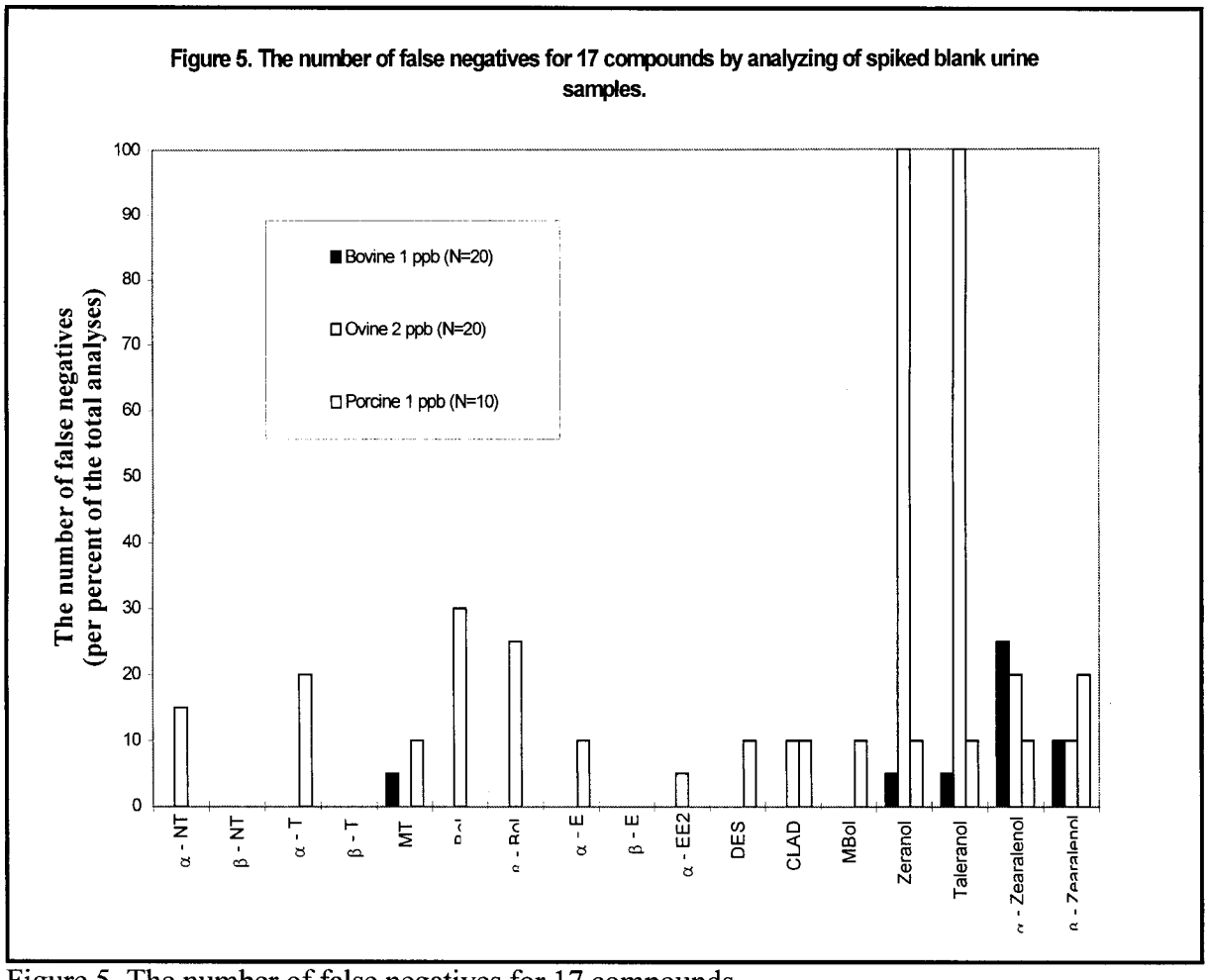
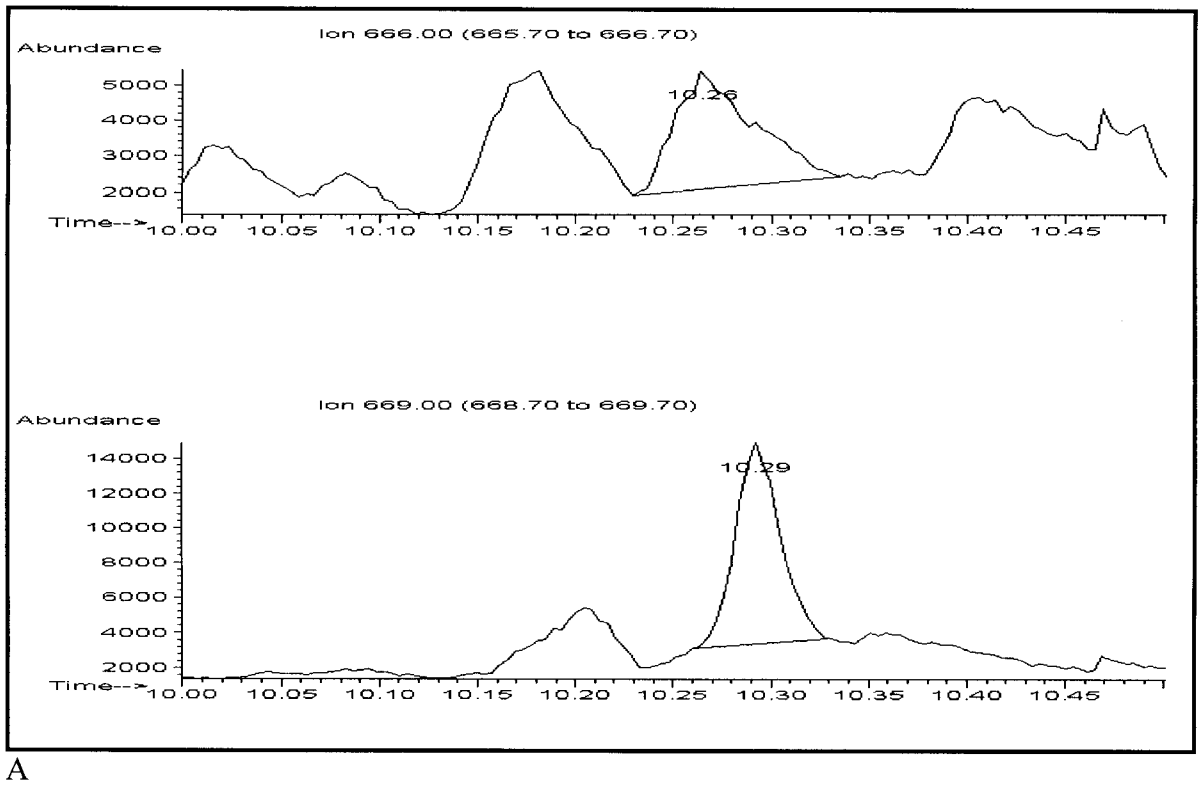


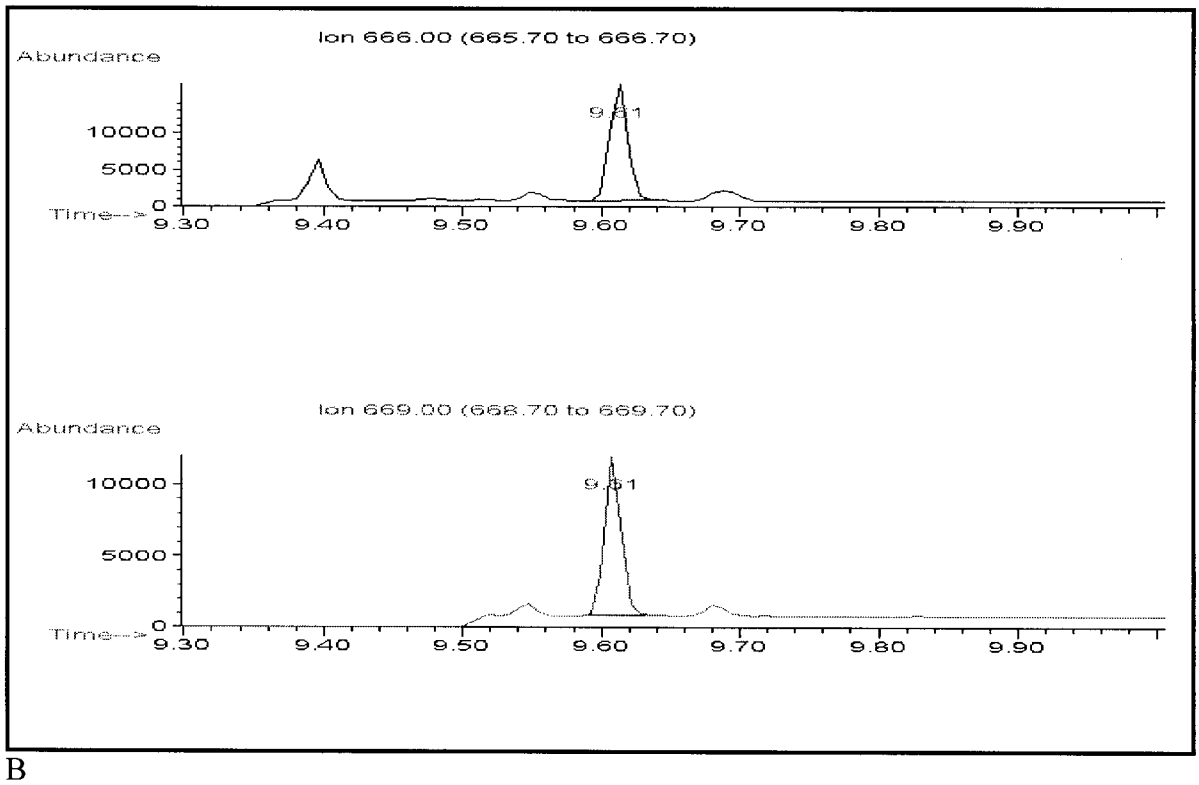
Figure 5. The number of false negatives for 17 compounds.

1.5.3 Influence of HPLC clean-up

In Figure 6 the effect of HPLC (Column-switching) is demonstrated. A comparison was made for clean-up only with ASPEC® and ASPEC® - coupled-column HPLC. From this Figure it becomes clear that the HPLC-step provides significant additional clean-up. A special problem encountered in the determination of anabolic compounds is the unique composition of every sample of urine. For some bovine- and porcine samples, clean-up with only SPE could be sufficient. In case of ovine urine samples, clean-up with SPE and coupled- column HPLC is necessary for all samples.



A



B

Figure 6[4]. Detection of 17ß-19-NT in a sample of ovine urine (a) without and (b) with coupled column HPLC procedure. Analytical conditions as decribed in the text.

[4]The difference in retention time between A and B is caused, by fact that the samples were not analysed on the same day.

Typical chromatograms obtained for a sample of bovine urine are presented in Figures 7 - 13. The concentration was 2 ppb for all compounds.

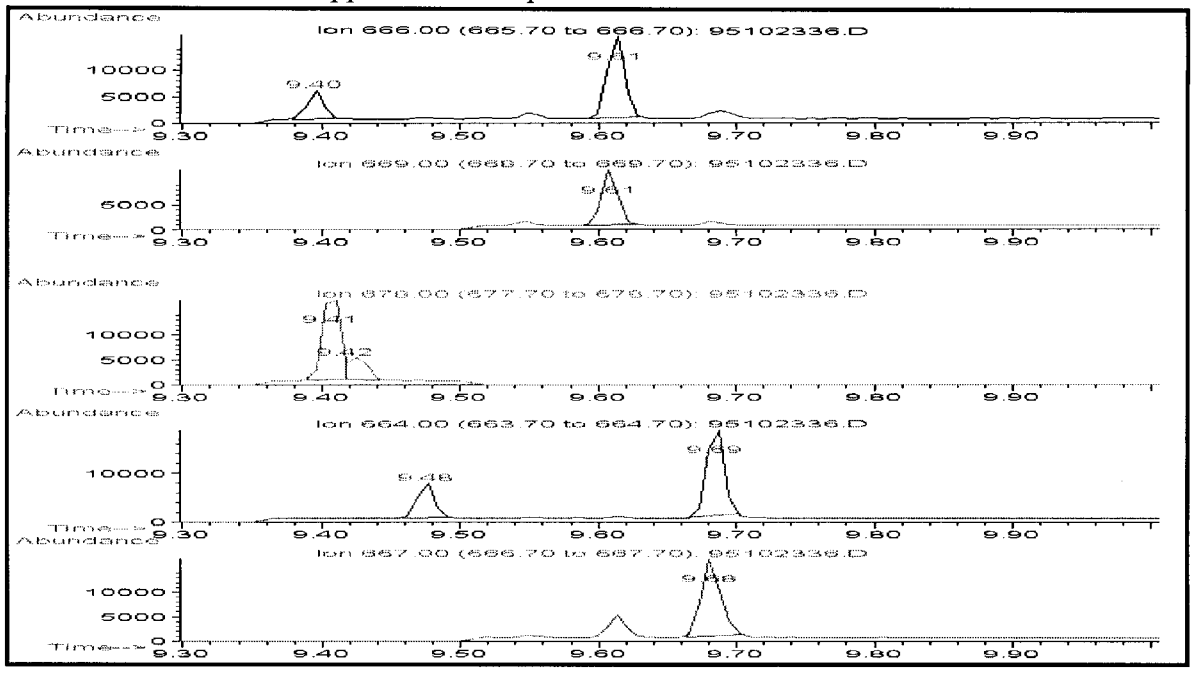


Figure 7. 17α -NT, 17ß-NT (m/z 666); 17ß-19-NTd3 (m/z 669), α -Bol, ß -Bol (m/z 678), 17α -E2, 17ß-E2 (m/z 664), 17ß-E2-d3 (m/z 667) (HFB - derivates)

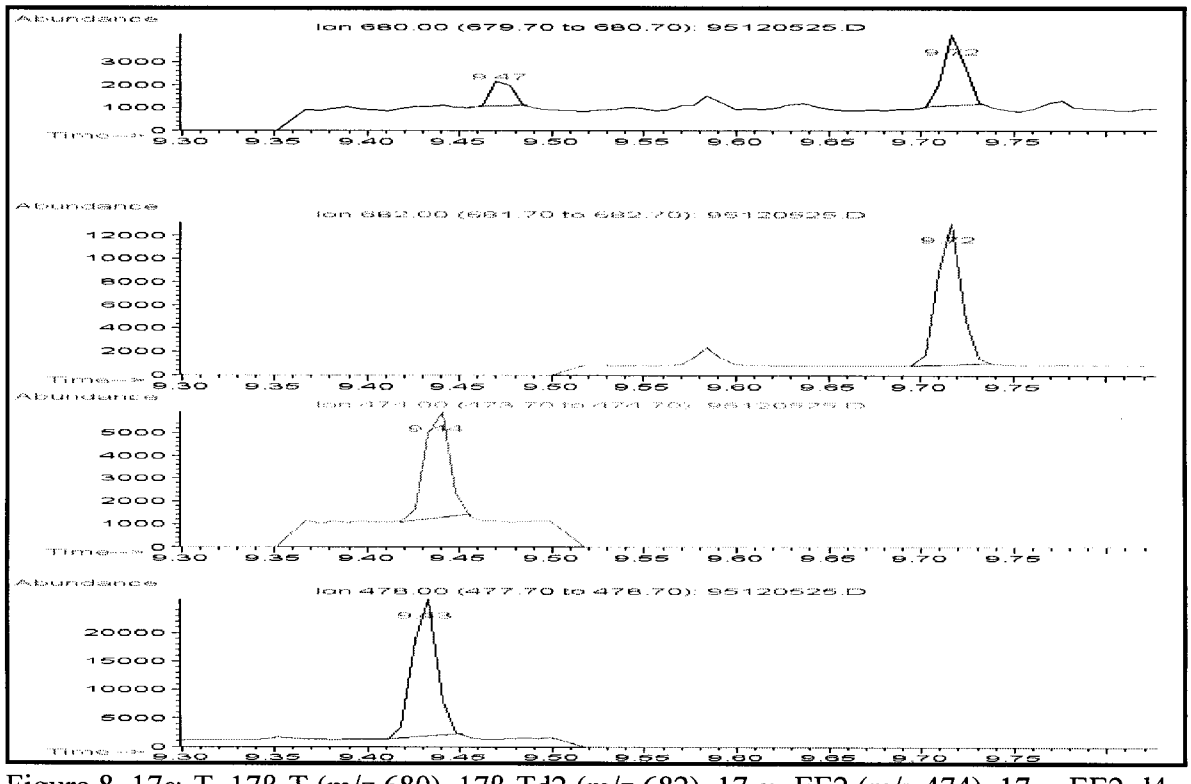


Figure 8. 17α-T, 17ß-T (m/z 680), 17ß-Td2 (m/z 682), 17 α -EE2 (m/z 474), 17 α-EE2-d4 (m/z 478). (HFB derivatives)

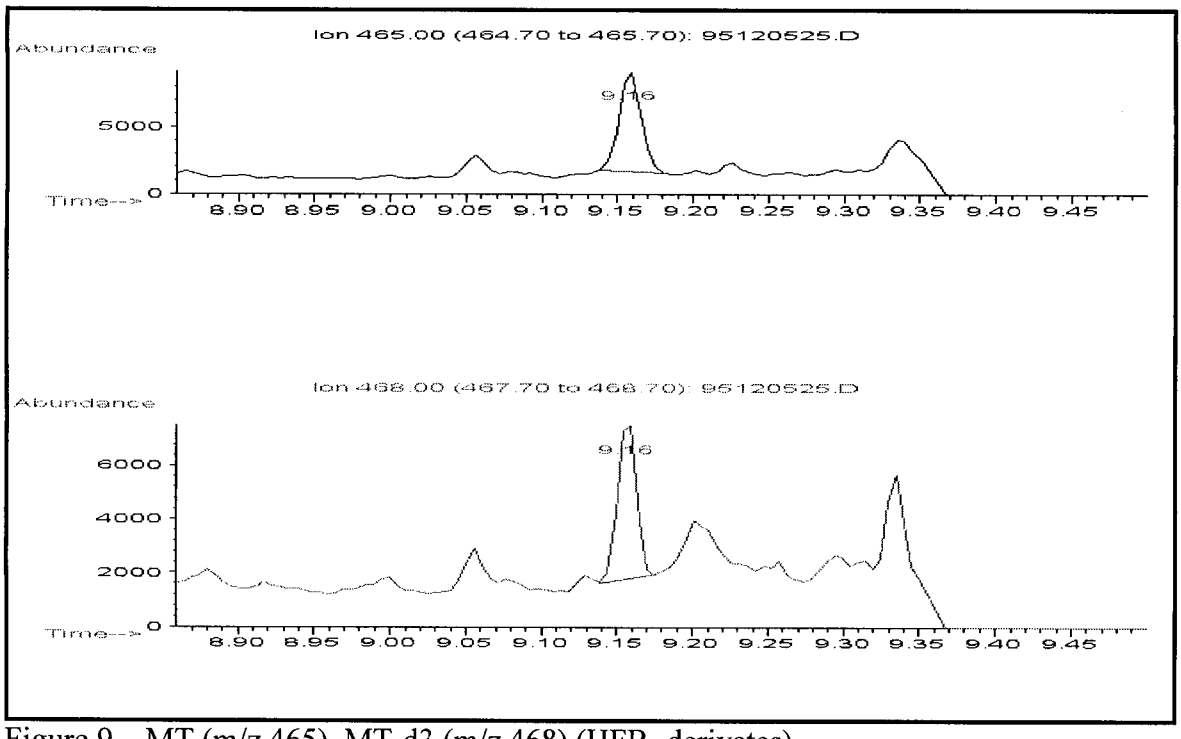


Figure 9. MT (m/z 465), MT-d3 (m/z 468) (HFB -derivates)

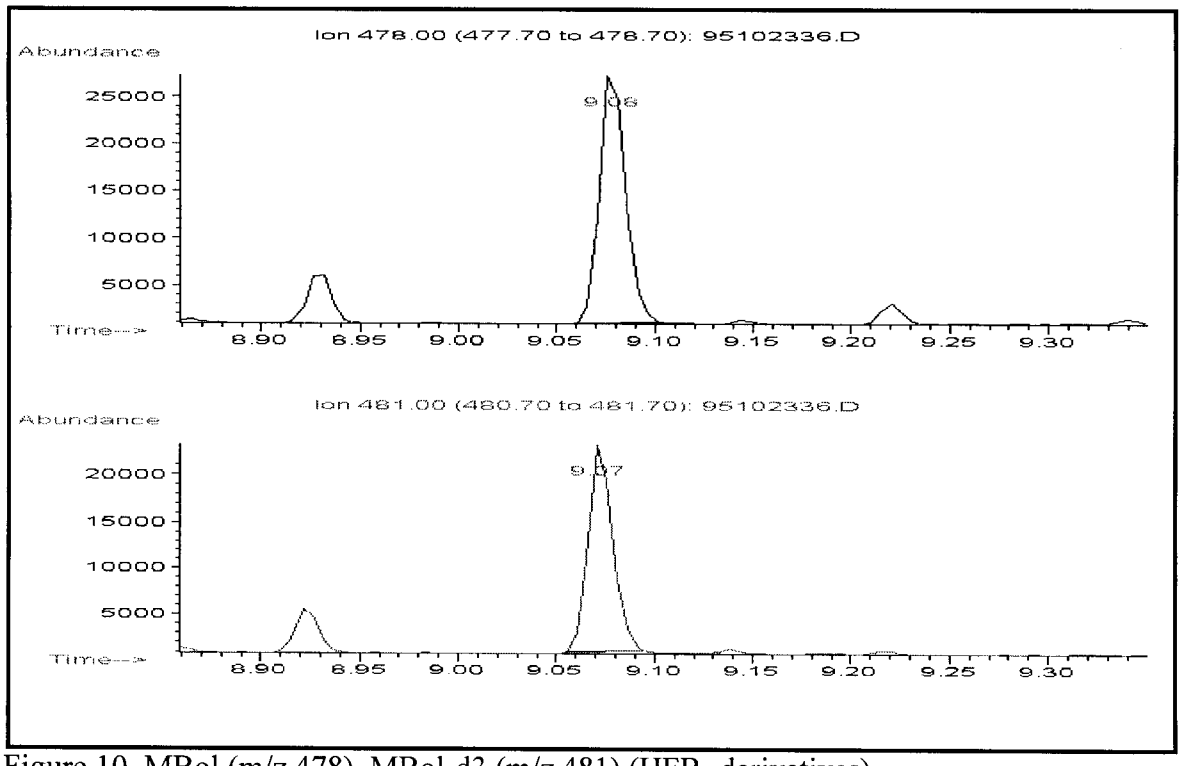


Figure 10. MBol (m/z 478), MBol-d3 (m/z 481) (HFB -derivatives)

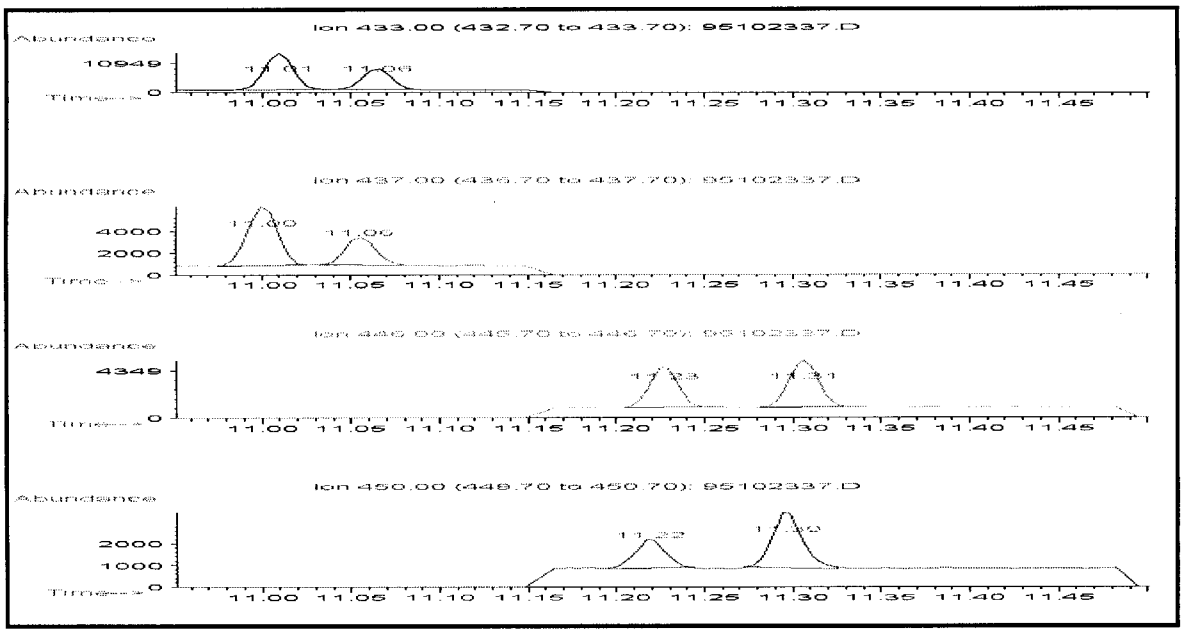


Figure 11. Zer, Tal (m/z 433), Zer-d4, Tal-d4 (m/z 437), α -Zer, ß -Zer (m/z 446), α -Zer-d4, ß-Zer-d4 (m/z 450). (TMS derivatives)

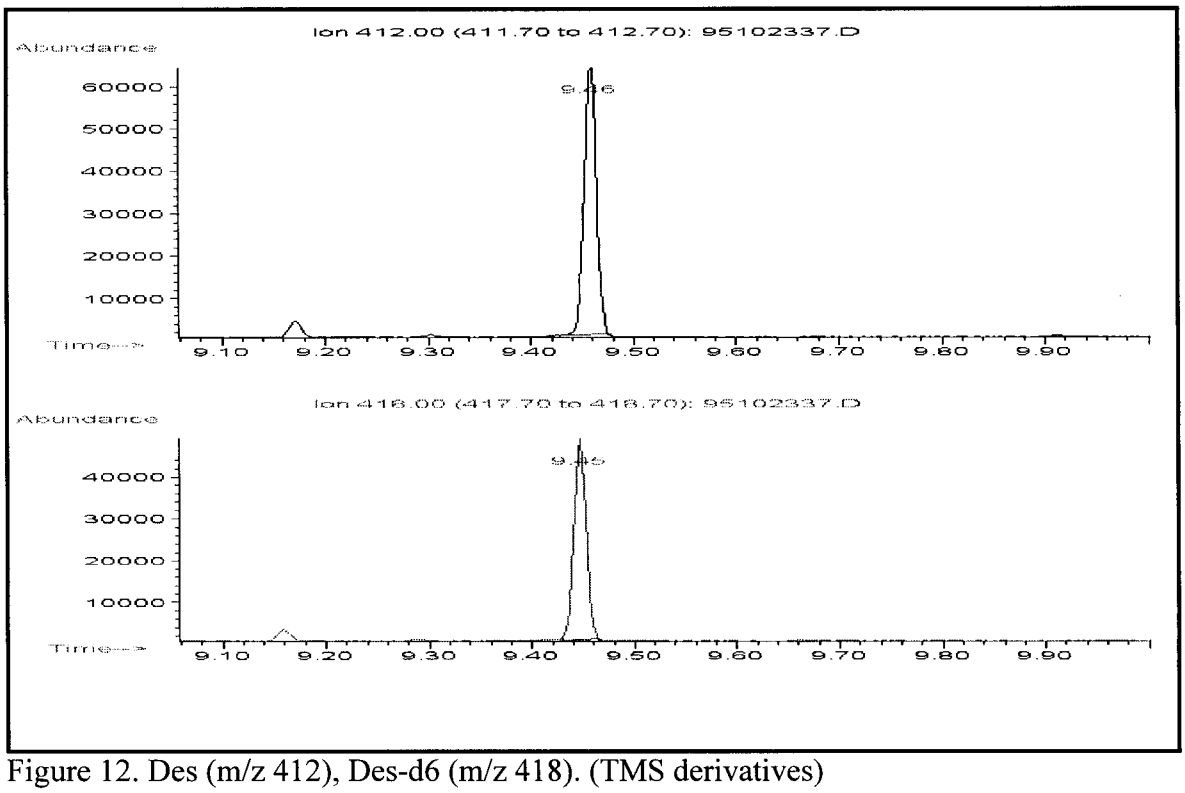


Figure 12. Des (m/z 412), Des-d6 (m/z 418). (TMS derivatives)

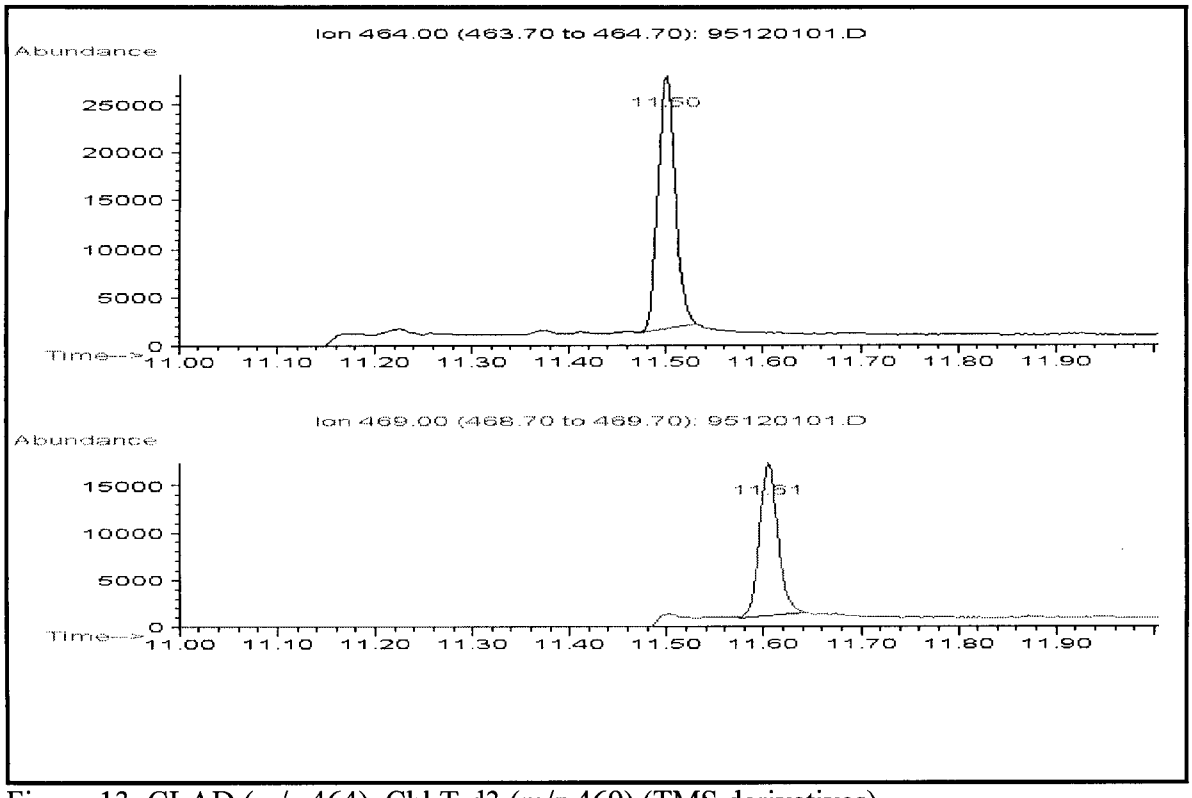


Figure 13. CLAD (m/z 464), Chl.T-d3 (m/z 469) (TMS derivatives)

1.6 Conclusions

The combination of SPE and coupled column HPLC can be used for clean-up of samples of urine from different species for screening at the 1 ppb level (2 ppb for ovine urine samples).

A comparison of the method with and without HPLC coupled - column clean-up step shows, that the extension with HPLC clean-up provides chromatograms with less interferences. The accuracy, detection limit and false negative results are acceptable for use of this method in residue control analysis, even though for a limited number of cases the set of performance criteria was not fulfilled. The fact that the method is automated contributes to a higher sample throughput.
The method was presented and demonstrated in a EU workshop in May 1996 (5).

2. Confirmatory analyses

2.1 Materials and Methods

The objective of the modified procedure described below was to separate the 17 analytes into a limited number of fractions. The amount of interferences within each fraction should be minimal, allowing the confirmation of the analytes in agreement with the EC/4 criteria . Figure 14 shows a schematic overview of the equipment used.

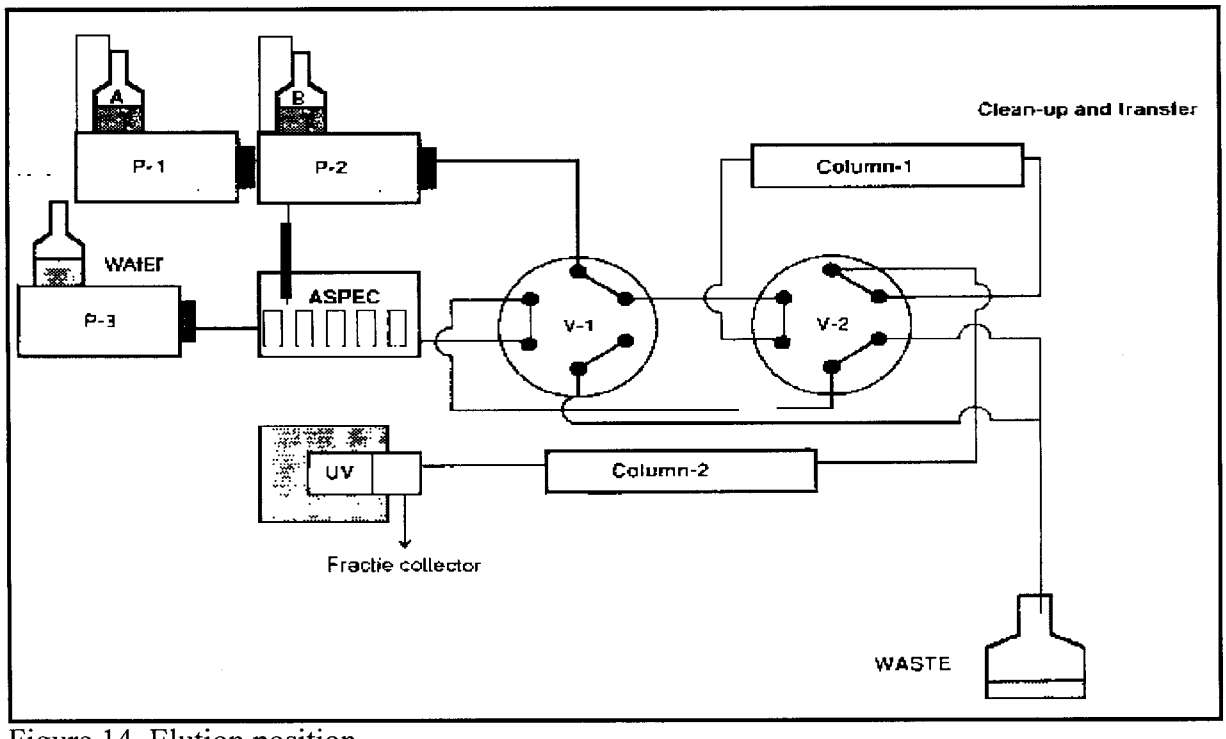


Figure 14. Elution position

Schematic setup of the coupled-column HPLC system for confirmatory analysis of anabolic agents in samples of urine. ASPEC® = Automated sample preparation with extraction columns; V-1 and V-2 = valves; P-1, P-2 and P-3 = LC pumps; UV = UV-vis detector, A = methanol/water (95:5) B = methanol/water (5:95).

2.2 Analytical procedure for confirmation

The overall scheme of the analytical procedure is given in Figure 15. Urine (10 ml), to which 40 µl Suc Helix Pomatia is added, is diluted after adjustment to pH=5.2, with 2 mol/l acetate buffer (1 ml) and spiked with the relevant internal standard. For confirmation of the identitiy a duplicate sample is analysed without internal standard in case of possible interferences between fragments of the internal standard and the analyte to be confirmed. The mixture is vortexed for 30 sec. and hydrolysed overnight at 37 °C. After hydrolysis the mixture is centrifuged for 5 minutes at 3600 rpm.

2.2.1 ASPEC® clean-up

The solid-phase extraction is performed automatically by a Gilson System equipped with disposable Bakerbond cartridges.
The procedure is as follows:

1. The C18 column is preconditioned with 5 ml of methanol and 5 ml of milli-Q water and the sample is loaded on the column. The column is washed with 5 ml of milli-Q water and a mixture of 4 ml methanol/milli-Q water (60:40) and the analytes are elueted with 3 ml of acetone.

2. The NH_2 column is preconditioned with a mixture of 5 ml of methanol/milli-Q water (60:40) and the collected eluate (3 ml acetone) from C18 is dispensed onto the amino column. This eluate is collected, reduced to approximately 1 ml and diluted with 1 ml of water.

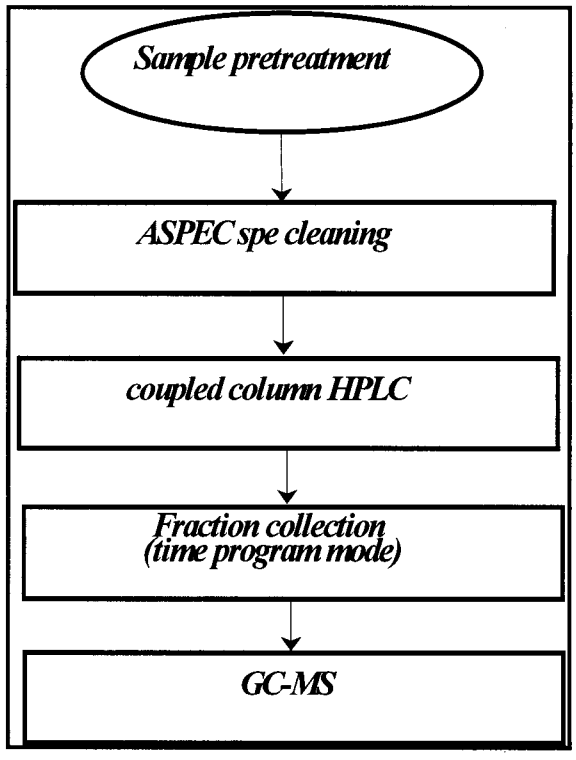


Figure 15. Overall scheme of the method.

2.2.2 Coupled-column HPLC

The total eluate (2 ml) is injected on column-1 of the HPLC system. For the first 4 minutes following injection, column-1 effluent is sent directly to the waste at a flow rate of 0.6 ml/min. (The eluent of pump P3 is water to remove the acetone). The next step is to wash column-1 for 4 minutes with a mixture of methanol/water (40:60) at the same flow rate. After column-1 is coupled, via a switching valve, to column-2 the analytes are eluted with a linear gradient from 95% mobile phase A (= mixture of water/methanol (95:5)) to 95% mobile phase B (= mixture of water/methanol (5:95)), at a flow rate of 0.6 ml/min.
The eluate is collected with a fraction collector. The correct moment to start collection, depends of the start/end of a peak, the volume of the tubing between the UV-detector and the fraction collector and the flow of the mobile phase. It is important to check the correct time of collection with an injection of a standard solution.
The first collection time is ± 20 minutes after column coupling. Three fractions of 8 minutes are collected. Figure 16 shows a chromatogram of Zer and Tal, α -Zer and β -Zer and DES eluting under the described conditions, obtained with UV detection. These compounds are representative for all compounds included in the method. The technique with gradient elution and column switching results in good separation for all 17 compounds. The LC clean-up with gradient elution gives better chromatograms than the LC clean-up with isocratic elution used in the method for screening.

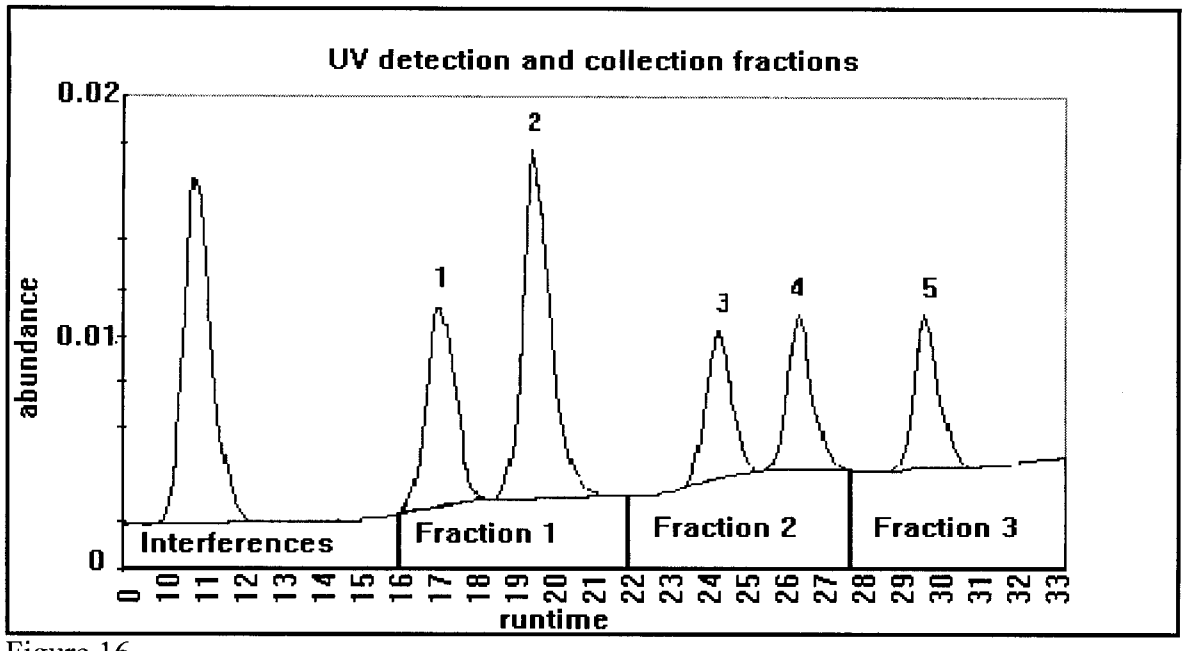


Figure 16.
Chromatogram of standard solution containing (1)Taleranol, (2)ß-Zearalenol, (3)Zeranol, (4)α-Zearalenol and (5)DES.

Figure 17 shows the gradient conditions and the collection windows of the system. The procedure consists of 5 steps: (after injection) loading the first column with water, washing the first column, (after column coupling) eluting the columns, fraction collection, reconditioning of the columns.

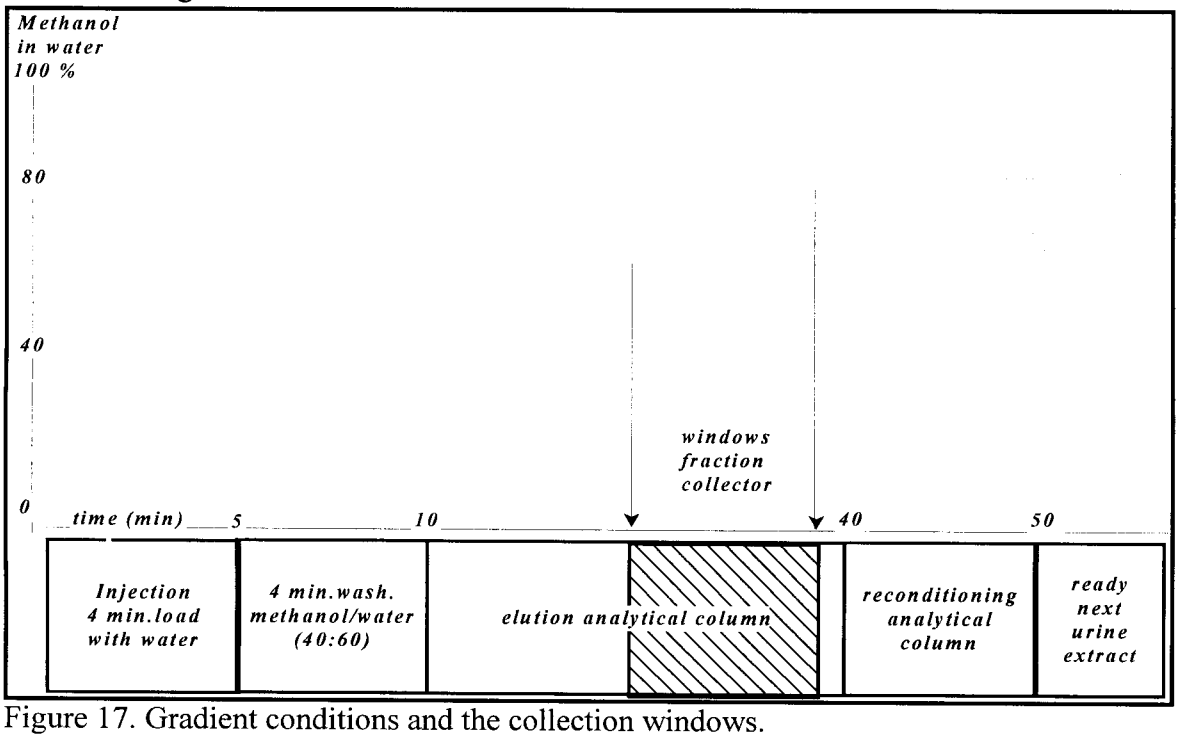


Figure 17. Gradient conditions and the collection windows.

The analytes included in this procedure and the retention times (indicative) of each compound are listed in Table 6. It can be seen that most anabolics are eluted in the range between 20 - 37 minutes. An UV-chromatogram of blank urine (Figure 18) shows that this time window is free of most of the matrix components.

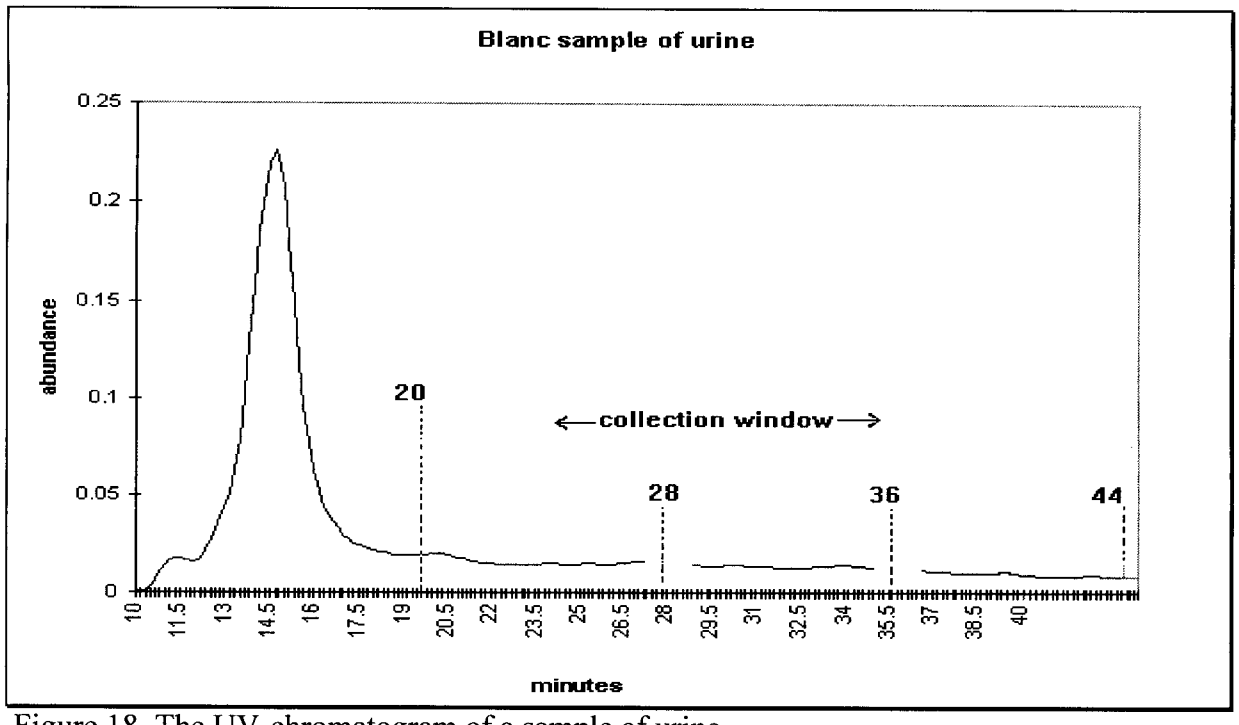


Figure 18. The UV-chromatogram of a sample of urine.

Table 5. HPLC retention times (indicative) and fractions.

Anabolic agents	Rtime (min.)	Fract. 1	Fract. 2	Fract. 3
CLAD	33.5		+	
DES	35.4			+
Zer	29.7		+	
Tal	20.7	+		
a-Zer	32.3		+	
ß-Zer	23.8	+		
17a-E	32.1		+	
17ß-E	32.1		+	
17ß-NT	28.4		+	
17a-NT	32.4		+	
17ß-Bol	24.9	+		
17a-Bol	30.3		+	
M-Bol	29.2		+	
MT	37.4			+
17ß-T	32.5		+	
17a-T	37.3			+
EE2	32.1		+	

2.2.3 Derivatization

The eluates are evaporated at 50 °C under a stream of nitrogen until dryness and dissolved in methanol (0.5 ml). The extract is transferred to a derivatization-vial and evaporated at 50 °C under a stream of nitrogen until dryness. The dry residue is derivatized with 25 µl of Heptafluorobutyric Acid Anhydride/acetone p.a. (1:4) or 20 µl of a mixture N-methyl-N-trimethylsilyltrifluoroacetamide(MSTFA)-ammoniumiodide-dithioerythritol(1000:2:4, v/w/w) depending on the analyte to be confirmed. The derivatization-vials are placed in a heater at 60 °C for 1 hour and the solvent is evaporated at 50 °C under a stream of nitrogen until dryness and the residue is dissolved in iso-octane (25 µl) and transferred to an injection-vial with micro insert. The 7673 automatic sampler injects 2 µl of the extract into the GC-MS. The samples are analysed for the presence of the diagnostic ions listed in Table 6.

2.2.4 GC-MS conditions

The gas chromatograph is equipped with a 60 meter CP SIL-5 (Low Bleeding/non polar) fused silica capillary column (0.25-mm i.d., 0.1 mm film thickness, SGE).
The oven temperature is held at 80 °C for 1 min, then increased at 30 °C/min. to 300 °C and held at this final temperature for 5 min. The injector temperature is maintained at 250 °C, the detector temperature at 250 °C for the MS-source and at 120 °C for the MS-quadrupool. The instrument is operated in the electron-impact ionization (EI) mode. The injection port is set in the splitless mode at injection and after 0.75 minutes in the split mode. The helium carrier gas flow rate is 0.6 ml/min. The Electronic Pressure Control (EPC) system is used for constant flow. Therefore, the pressure is held at 50 psi for 1 minute, then decreased at 99 psi/minute to 28.4 psi. Finally, the flow is regulated through the system for constant flow, during the oven-temperature program.

2.3 Results

The ratios between 4 diagnostic ions are calculated by dividing the response of a fragment-ion with the response of the most intense fragmention. The ratios in Table 6 are obtained by injecting 100 ng of a standard, after clean-up and derivatization with the method described above. These ratios only can be used as an indication since many experimental parameters are of influence on the actual values observed.

Table 6. The ratios for each compound calculated by injection of a standard analysed.

Anabolic agents	4 Diag. ions (underl.= most abundant)	Rtime (min)	Ratio 1	Ratio 2	Ratio 3	Derv
17α-E2	664, 451, 409, 356	9.48	2.617	1.854	3.500	HFB
17ß-E2	664, 451, 409, 356	9.68	2.351	1.933	1.466	HFB
17α-NT	666, 453, 306, 133	9.39	1.299	0.709	1.520	HFB
17ß-NT	666, 453, 306, 133	9.6	1.015	1.053	1.192	HFB
α-Bol	678, 464, 369, 169	9.42	11.64	3.985	7.980	HFB
ß-Bol	678, 464, 369, 169	9.39	4.172	2.171	5.081	HFB
M-Bol	478, 463, 435, 367	9.07	1.028	0.387	1.540	HFB
MT	480, 465, 369, 355	9.15	1.276	0.418	0.344	HFB
17α-T	680, 467, 355, 320	9.46	1.409	0.631	0.976	HFB
17ß-T	680, 467, 355, 320	9.68	1.645	0.561	1.901	HFB
17ß-EE2	474, 459, 446, 353	9.41	0.930	1.073	0.415	HFB
CLAD	464, 392, 357, 221	11.54	0.039	0.017	0.104	TMS
DES	413, 412, 397, 383	9.45	0.350	0.193	0.290	TMS
Zer	538, 433, 335, 307	11.04	0.029	0.459	0.596	TMS
Tal	538, 433, 335, 307	11.09	0.037	0.550	0.719	TMS
α-Zer	536, 446, 333, 305	11.26	0.141	0.884	1.697	TMS
ß-Zer	536, 446, 333, 305	11.35	0.234	1.027	1.512	TMS

2.3.1 Confirmation

To test the method, 8 samples of blank porcine urine, obtained from the "Bank of Reference Blank Samples" were spiked with one or more of the following compounds 17α-T, 17ß-T, 17α-NT, 17ß-NT, MT, 17α-E2, 17ß-E2, 17ß-EE2, DES, CLAD, 17α-Bol, 17ß-Bol, MBol, α-Zer, ß-Zer, ZER and TAL at the level of 2 ppb. The ratios were calculated and compared with the ratios obtained for the standards (Table 6 and 7). The deviation from the ratio obtained with the standard is given as percentage.
It can be seen that for nearly all compounds the EC-criteria (± 10%) are fulfilled. It has to be kept in mind that this experiment was performed with spiked samples of urine. The compounds were not conjugated (enzymes for hydrolysis were added to the urine). In routine analysis every urine can be different. For evaluation the amount of an analyte and the standard have to be in the same range. Moreover, multiple injections frequently are necessary to come to a final conclusion with respect to the identify of the compound.
The results are summarized in Table 7.

Table 7. Ion ratios obtained for spiked samples of porcine urine at 2 ppb level.

Anabolic agents	Ret.time	Ratio 1	% *	Ratio 2	% *	Ratio 3	% *
CLAD	11.54	0.053	**36**	0.016	6	0.099	5
DES	9.45	0.345	1	0.190	2	0.334	**15**
Zer	11.04	0.026	10	0.436	5	0.597	0
Tal	11.09	0.035	5	0.564	3	0.695	3
α-Zer	11.26	0.144	2	0.902	2	1.688	1
ß-Zer	11.35	0.234	0	0.919	**11**	1.465	3
17α-E2	9.48	2.217	**15**	1.647	**11**	2.763	**21**
17ß-E2	9.68	2.604	**11**	2.277	**18**	1.713	**17**
17α-NT	9.39	1.292	1	0.810	**14**	1.662	9
17ß-NT	9.6	1.084	7	1.389	**32**	1.644	**38**
α-Bol	9.42	9.914	**15**	3.651	8	7.158	10
ß-Bol	9.39	4.515	8	2.175	0	5.237	3
M-Bol	9.07	0.951	7	0.372	4	1.613	5
MT	9.15	1.203	6	0.498	**19**	0.387	**13**
17α-T	9.46	1.333	5	0.660	5	1.051	8
17ß-T	9.68	1.666	1	0.701	**25**	2.406	**27**
17ß-EE2	9.41	0.943	1	1.076	0	0.411	1

* = Deviation from ratio of a standard.

2.4 Conclusions

The procedure for confirmation, derived from the previously described procedure for screening, is suitable for identifying residues detected. However, as with most procedures, additional analyses will frequently be necessary for full confirmation according to all criteria (4,10).

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