1. Introduction

This method of analysis describes the detection and confirmation of the presence of 17ß-estradiol (17ß-E2) in samples of serum. Clean-up and concentration is done with extrelut®-columns and immunoaffinity chromatography (IAC) or solid phase chromatography (SPE). After derivatization with HFBA 17ß-estradiol is analyzed with GC-MS (Gas Chromatography-Mass Spectrometry). For purposes of quantification a deuterated internal standard 17ß-Estradiol-d3 (I.S. 17ß-E2-d3) is available.

The method is used to perform routine screening and confirmatory analyses in bovine serum. The limit of detection is 20 ng/l or ng/kg, based on the detection of the most abundant diagnostic ion. The limit of determination is 40 ng/l or ng/kg.

2. Theory

2.1. Description of 17ß-Estradiol

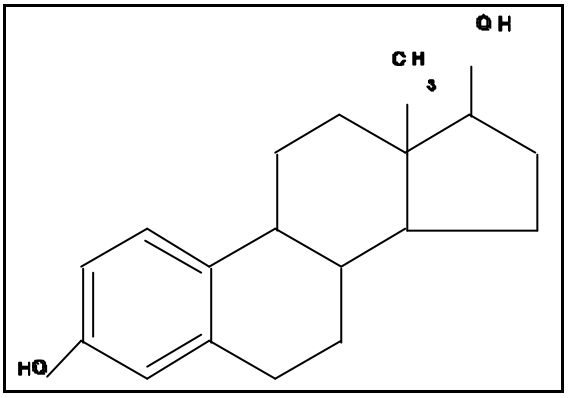

Figure 1 : Structure 17ß-Estradiol

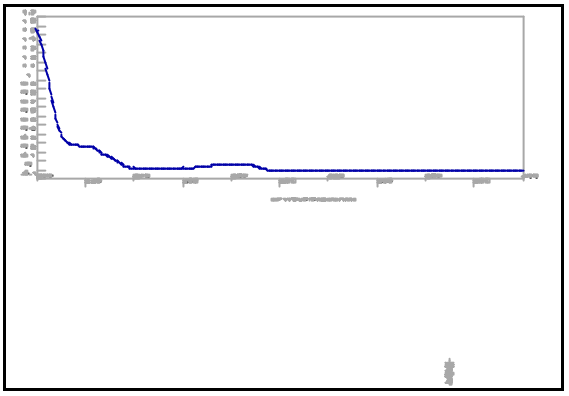

Figure 2 : UV - spectrum of estradiol

17ß-Estradiol (17ß-E2), Estra-1,3,5(10)-triene-3,17ß-diol ($C_{18}H_{24}O_2$)
Molecular Weight (MW): 272.4, CAS(Chemical Abstracts Service)-number: 50-28-2

White or creamy-white, odourless, hygroscopic crystals or crystalline powder. Practically insoluble in water; soluble in alcohol (1 in 28), chloroform (1 in 435), ether (1 in 15), soluble in acetone, dioxane, and solutions of fixed alkali hydroxides, sparingly soluble in vegetable oils.
17ß-Estradiol is a naturally occurring oestrogenic hormone, especially present in female animals. It can also be synthesized. Natural and synthetic hormones (steroids) are used worldwide as growthpromoting agents in animal breeding. The use of these substances for this purpose is prohibited within the European Union (EU).

2.2. Extrelut®

An aqueous sample is applied to a column filled with granular support material (=extrelut®), and becomes distributed to form the stationary phase on the porous matrix. Elution of the column is carried out using organic solvents not miscible with water (ethyl acetate). Lipophilic compounds are extracted by the solvent from the aqueous phase in the process and quantitative elution takes place. The eluate does not contain emulsions.

The substances dissolved in the eluate are examined either immediately or following purification and/or concentration. The pH of the aqueous solution to be extracted may be in the range from 1 to 13. This enables separations of acid, neutral and alkaline compounds.

2.3. Extract clean-up

Two different extract clean-up procedures can be used: IAC or SPE. Both procedures give similar results. At the RIVM (Rijksinstituut voor Volksgezondheid en Milieu; National Institute of Public Health and the Environment) the IAC procedure is used, but the SPE procedure can be used alternatively.

IAC

Immunoaffinity chromatography is a special form of affinity chromatography. It can be used for purification in residue analysis because of it's high selectivity. The antiserum is obtained by immunizing rabbits. The immunoglobulin-G fraction (IgG), which is needed for the preparation of IAC columns, is isolated from rabbit antiserum and coupled to a Sepharose base with Protein A (See SOP ARO/172, Preparation of IAC-columns).

SPE

Solid Phase Extraction is a sample preparation technique based on the separation mechanisms of liquid chromatography (LC). In LC, the solubility and functional group interactions of samples, sorbent and solvent are optimized to achieve a separation. In solid phase extraction these interactions are optimized to achieve retention or elution. SPE-steps are conditioning, sample application, washing and elution.

2.4. Derivatization

For GC-MS analysis of 17ß-estradiol several derivatives can be made. Because of the two hydroxyl-groups (see figure 1) both TMCS-derivatives (trimethylchlorsilan) and HFBA-derivatives (heptafluorobutyric acid anhydride) can be prepared. HFBA-derivatives of 17ß-estradiol give cleaner chromatograms. Therefore derivatization with HFBA is chosen for analysis of 17ß-estradiol in serum. The molecular weight of 17ß-estradiol-di-HFB is 664. Figure 3 shows the reaction of this derivatization.

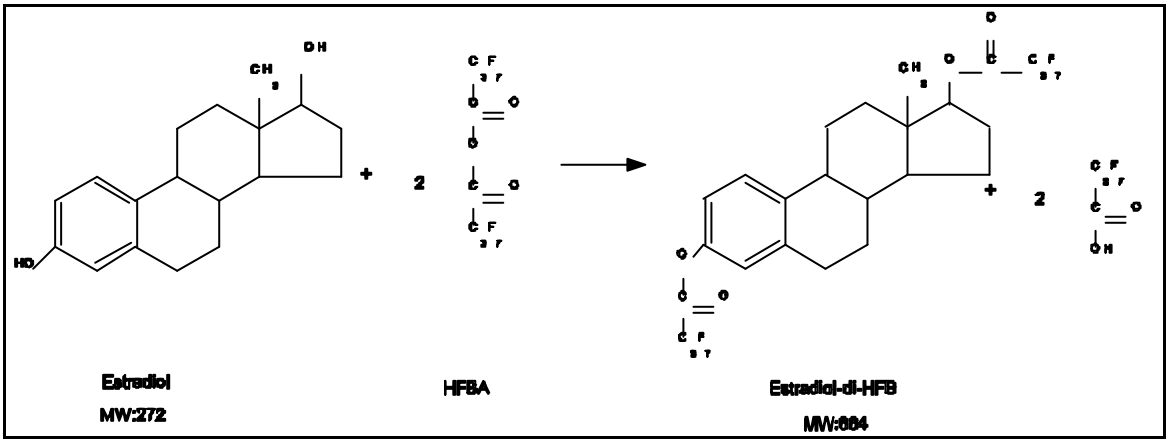

Figure 3 : HFBA-derivatization of estradiol

2.5. Mass spectrometry

Several ionization techniques are available. Most frequently used is **electron impact** (EI). This technique produces ions for mass spectrometry by bombardment of the gaseous sample with a beam of energetic electrons (70 eV). This causes extensive fragmentation leading to a large number of positive ions of various masses smaller than that of the original derivative. The complex mass patterns are useful for identification.

Figure 4 shows the EI-spectrum of 17ß-estradiol (17ß-E2-di-HFB-derivative).

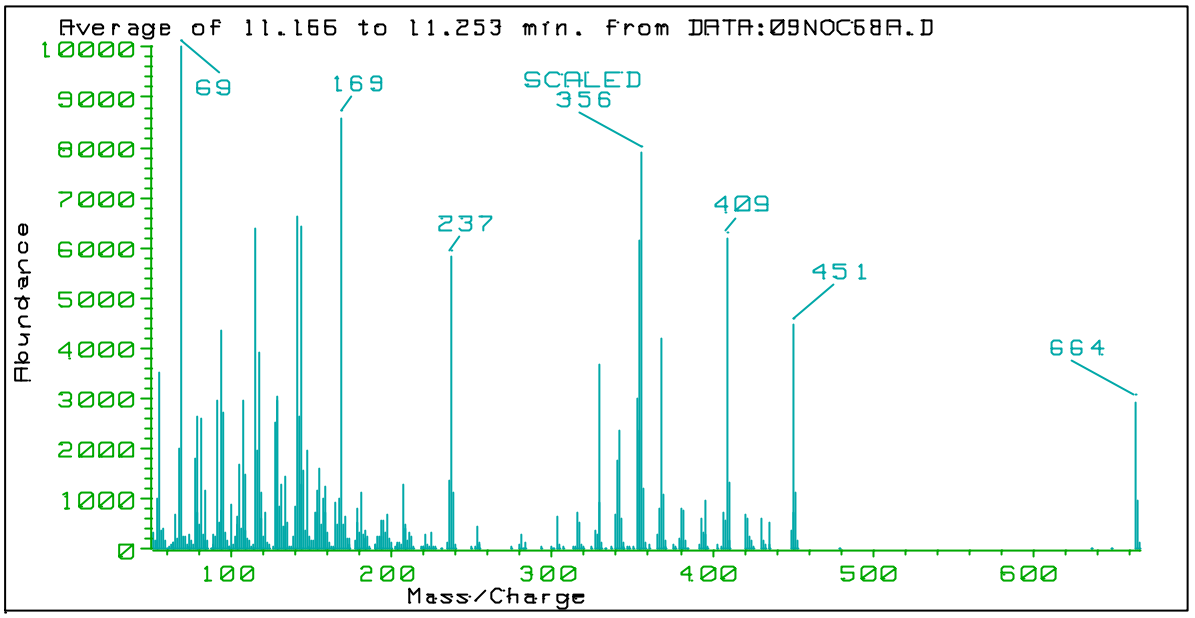

Figure 4 : GC-MS spectrum of Estradiol-di-HFB (EI)

Diagnostic ions: m/z (mass/charge) 664 (basepeak), 451, 409, 356

3. Materials

3.1 Standards

For quantification the use of reference standards is recommended. Ampoules of 17ß-estradiol and 17ß-estradiol-d3 (internal standard) are prepared at the RIVM each containing 0.1 mg per ampoule (SOP ARO/374).
17ß-Estradiol was obtained from Diosynth/Organon (RIVM/ARO H145819)
17ß-Estradiol-d3 was obtained from MSD isotopes/Sanbio (RIVM/ARO 89M1691).

Table 1: Standards included in this SOP.

analyte	abbreviation	CAS-number	formula	mol.weight	RIVM code ampoule
17ß-Estradiol	17ß-E2	50-28-2	$C_{18}H_{24}O_2$	272.4	92M3779
17ß-Estradiol-d3	17ß-E2-d3	-	$C_{18}H_{21}O_2D_3$	275.4	93M0028

From these standards a stock solution containing 1 mg/ml is prepared in ethanol. This so- lution is registered and stored in the dark at approximately -20? C (not higher than -10? C).

Working solutions are prepared by ten-fold dilution of the stock solution (down to 0.01 ng/µl). Solutions of 1 mg/ml and 100 ng/µl are stored for a maximum period of 5 years (-20? C). Lower concentrations are stored in the dark at approximately 4? C (range 1-10? C) for a maximum period of 6 months. Quality control includes the registration of a mass spectrum (identity) and a HPLC Diode Array chromatogram/UV-spectrum of the reference standard ampoules.

3.2 Samples

Samples are stored in the dark at approximately -20? C, but not higher than -10? C, until analysis, or at approximately 4? C (range 1 - 10? C) if analysis is foreseen within 2 days.

3.3 Chemicals

3.3.1 17ß-Estradiol (Diosynth/Organon; RIVM/ARO H145819)
3.3.2 17ß-Estradiol-d3 (MSD isotopes/Sanbio; RIVM/ARO 89M1691).
3.3.3 Ethanol p.a. (Baker, art. 8006).
3.3.4 Ethyl acetate p.a. (Baker, art. 8037).
3.3.5 HFBA (Heptafluorobutyric acid anhydride; Pierce, art. 63164).
3.3.6 BSTFA + 1% TMCS (N,O-Bis(trimethylsilyl)trifluoracetamide + 1% trimethylchlorsilan; Pierce, art. 38832).
3.3.7 Acetone (Romil, Hi-dry, art. D4032).
3.3.8 Iso-octane (Baker, art. 8715).
3.3.9 Disodium hydrogen phosphate (Merck, art. 6586).
3.3.10 Potassium dihydrogen phosphate (Baker, art. 0240).
3.3.11 Sodium chloride (Baker, art. 0278).
3.3.12 Thiomersal (BDH, art. 304162).
3.3.13 Methanol p.a. (Baker, art. 8402).
3.3.14 Phosphate buffer 0.02 mol/l, pH 7.4. Dissolve in 800 ml of water:
2.278 g disodium hydrogen phosphate,
0.416 g potassium dihydrogen phosphate,
9.0 g sodiumchloride,
0.05 g thiomersal.
Adjust the pH at 7.4 ? 0.1 and add water to a final volume of 1000 ml.
3.3.15 IAC-eluting solution (Ethanol/H_2O, 50:50): Add to 50 ml of ethanol, water to a final volume of 100 ml.
3.3.16 IAC-washing solution (Ethanol/H_2O; 80:20): Add to 80 ml of ethanol, water to a final volume of 100 ml.
3.3.17 SPE-washing solution (Methanol/H_2O; 45:55): Add to 45 ml of methanol, water to a final volume of 100 ml.
3.3.18 SPE-eluting solution (Methanol/H_2O; 80:20): Add to 80 ml of methanol, water to a final volume of 100 ml.

Notice:
-Solutions are aqueous solutions with bi-distilled water
-Reference to a company and/or product is only for identification and information and does not imply approval or recommendation of the company and/or product of the mentioned company and/or product or an exclusion of other compagnies and/or products by the RIVM.

3.4 Apparatus

Standard laboratory glassware and equipment is used, with in addition:
3.4.1 Flatbottom flasks, 150 ml (Quickfit, art. FF150/4S)
3.4.2 Glass tubes 50 ml (Corex U.S.A., art. 8422-A)
3.4.3 Glass vials, 20 ml with screw cap (Packard)
3.4.4 Centrifuge tubes, glass (55 mm x 11.5 mm, RB55 ; Renes)
3.4.5 Extrelut®-20 columns (Merck, art. 11737)
3.4.6 IAC-columns (17ß-Estradiol/17ß-Testosterone IAC column; RIVM/ARO; 91M3497)
3.4.7 Evaporation block (Thermolyne, type 16500 - or 17600 - Dri bath) with nitrogen facility

3.4.8 Glass derivatization vials (Chromacol 2SV[A]) with screw caps (Chromacol 8SC) and septa (Chromacol 8-ST15)
3.4.9 Glass injection vials (Alltech, art. 98239) with crimp caps (Alltech, art. 73071) and inserts (Alltech, art. 06090357)
3.4.10 SPE column (SEP-Pak C18 cartridges, Waters, art. 51910)
3.4.11 GC-MS equipment:
Gas chromatograph (Hewlett Packard, type 5890 II), split/splitless injection,
GC capillary column; BPX-5; non polar, fused silica, I.D: 0.22 mm, 50 m, film 0.25 µm, (SGE, art. 054114),
Automatic injector (Hewlett Packard, type 7673 GC/SFC injector),
Mass spectrometer (Hewlett Packard, type 5989A, Engine) with EI, PCI, NCI, DIP, HED,
Controller (Hewlett Packard, type 7673 controller),
Cooling unit for MS-system (NESLAB, Coolflow CFT-75),
Computer (Vectra 486) with workstation (HP MS Chemstation; G1034C, version C.02.05),
Printer (HP Laserjet 4),
Tape streamer (Jetstore 2000, Arcsolo software).

Notice:
-For operating instructions and maintenance status files see ARO/MIS data-bases: CB\AMAP and CB\INVENTAR.
-Reference to a company and/or product is only for identification and information and does not imply approval or recommmendation of the company and/or product of the mentioned company and/or product or an exclusion of other compagnies and/or products by the RIVM.
-The GC-MS is equiped with a High Energy Dynode (HED). This gives higher sensitivity. A HighMass autotune is performed to increase sensitivity for higher masses.

4. Analytical procedure 17ß-Estradiol in (lyophilized) serum

4.1. Sample preparation

The sample is or lyophilized serum or serum. Start the procedure at 4.1.1 if the sample is lyophilized serum (RIVM code: 94M2604 - 94M2607). If the sample is serum start at 4.1.6.

Lyophilized serum

4.1.1 Open 10 sealed ampoules (1 ml lyophilized serum per ampoule).
4.1.2 Add 0.5 ml water to each ampoule, mix with a Vortex mixer, and pipette the solutions of the ten ampoules in a single Corex glass tube
4.1.3 Repeat 4.1.2 two times, (total volume in the tube approximately 15 ml)
4.1.4 Add water to the glass tube to a total volume of 20 ml (= maximum capacity for extrelut®- columns).
4.1.5 Go to 4.1.8.

Serum

4.1.6 Pipette 10 ml of serum in a clean Corex tube
4.1.7 Add water to a total volume of 20 ml (= maximum capacity for extrelut®-columns)
4.1.8 Add internal standard: 200 µl of 0.01 ng/µl 17ß-E2-d3 (=2.0 ng absolute)
4.1.9 Mix with a Vortex mixer for 30 seconds
4.1.10 Mix for 30 minutes with a Rotation apparatus at room temperature

4.2 Extrelut®

4.2.1 Apply the sample to an extrelut®-column
4.2.2 Wait for 15 minutes
4.2.3 Add 60 ml of ethyl acetate into the extrelut®-column
4.2.4 Collect the eluate in a 150 ml flatbottom flask
4.2.5 Evaporate the Ethyl acetate with a rotation evaporator
4.2.6 Dissolve the sample in 500 µl of ethanol and mix with a Vortex mixer
4.2.7 Add 10 ml water into the 150 ml flatbottom flask

4.3 IAC or SPE

Immuno Affinity Chromatography (IAC)

4.3.1 Wash the IAC column with 20 ml water
4.3.2 Apply the sample of 4.2.7. on the IAC-column
4.3.3 Wash with 4 ml of water
4.3.4 Elute with 7 ml of a mixture of ethanol/water (1:1)
4.3.5 Collect the eluate in a clean centrifugetube
4.3.6 Wash the IAC column for re-use with 10 ml of a mixture of ethanol/water (8:2), wash with 20 ml water, store the columns in phosphate buffer at +4? C
4.3.7 Go to 4.3.13.

Solid Phase Extraction (SPE)

4.3.8 Precondition the SPE-column with 2 ml of methanol and 5 ml of water
4.3.9 Apply the sample of 4.2.7. to the SPE-column
4.3.10 Wash with 5 ml of water
4.3.11 Wash with 5 ml of a mixture of methanol/water (45:55)
4.3.12 Elute with 5 ml of a mixture of methanol/water (8:2) and collect in a clean centrifugetube.
4.3.13 Evaporate the eluate (IAC: 4.3.7. or SPE: 4.3.12.) on an evaporation block (or waterbath) at 50? C to dryness (under nitrogen)
4.3.14 Redissolve the residue in 500 µl of ethanol and mix with a Vortex mixer (0.5 min), place the centrifugetube in an ultrasonic waterbath (1 minute), and mix with a Vortex mixer for 15 seconds.

4.4 Derivatization

4.4.1 Transfer the solution 4.3.14. to a derivatization vial
4.4.2 Transfer aliquots of the standard solution 17ß-estradiol (corresponding to: 0, 0.2, 0.4, 1.0, 2.0, 4.0 ng absolute) directly into a series of derivatization vials
4.4.3 Add to tis series vials 2.0 ng 17ß-estradiol-d3 (=internal standard; similar to 7.1.8.)
4.4.4 Evaporate to dryness on an evaporation block under nitrogen at 50? C
4.4.5 Derivatize with 25 µl HFBA/Acetone (1:4) for 1 hour at 60? C in an oven
4.4.6 Evaporate to dryness under nitrogen on an evaporation block
4.4.7 Redissolve in 25 µl iso-octane
4.4.8 Inject 5 µl on the GC-MS (EI-mode), see appendix 1 for GC-MS method (using electronic pressure control during injection)
4.4.9 Monitor masses m/z: 664 (17ß-E2-di-HFB) and m/z: 667 (17ß-E2-d3-di-HFB)

5. Interpretation and calculation

5.1. Checks

The first step in interpreting the results is to check for:
- adequate performance characteristics of the GC-MS system (MS-tuning).
- adequate sensitivity for external derivatized standards (S/N 17ß-E2-d3 [2.0 ng absolute] > 15).
- adequate signals for internal standard in sample (S/N 17ß-E2-d3 [0.2 µg/l] > 15)

5.2. Calculation of quantitative results

Quantitative results are obtained by constructing calibration curves of the response variable (see 5.4.) versus the concentration. Quantification is only valid if:
- the maximum of the signal originating from the analyte has a S/N ratio > 6.
- the coefficient of correlation of the constructed calibration curve is better than 0.99
- the numerical value of the intercept does not deviate more than ? 3 SD from zero.

Calibration curves are calculated using least squares linear regression analysis.

5.3. Calculation in case of deuterated internal standards

When for the analyte concerned the corresponding deuterated internal standard was added to the test portion, quantification is straight forward. The area of the selected ion of the standard and internal standard are calculated and *the ratio is the response variable*. A calibration curve is constructed by analyzing different concentrations of standard in range of the inspected concentration in the sample. A linear curve is fitted using least squares linear regression calculation. Unknown concentrations are calculated by interpolation.

5.4. Identification

For identification according to the EC-criteria it is mandatory that the GC retention time corresponds with the retention time of the internal standard and that at least 4 ions are monitored. Each ion monitored (response) should fulfil the criterion that the S/N ratio of the maximum exceeds a value of 3. If this criterion is fulfilled the 3 different ratios are calculated. The same ratios are calculated for the standard analyte, preferably at the corresponding concentration. For positive identification the ratio obtained for the unknown sample should be within ? 10% of the average ratio value of the standard.

Notice:
Frequently the variability of the MS-equipment does not allow this criterion to be strictly applied. In this case the criterion is replaced by a criterion based on the experimental variability. The response ratios for the unknown sample should be within the corresponding standard value ? 3 SD. The validity of this approach was subject of discussion within the Benelux Public Health working group "Hormones and anti-Hormones"

6. Validation of the procedure

The repeatability of the method is < 10% (relative standard deviation). The within laboratory reproducibility is < 15%. Both values were determined at a level of approximately 0.45 µg/l. Further details are described in report 389002.019.

7. Related documents

7.1 Van Ginkel, L.A., Stephany, R.W., van Rossum, H.J., Steinbuch, H.M., Zomer, G., van de Heeft, E. and De Jong, A.P.J.M. (1989),
Multi-immunoaffinity Chromatography: A simple and highly selective clean-up method for multi anabolic residue analysis of meat,
J. Chromat. 489,111-120.

7.2 SOP ARO/172, Bereiding IAC-kolommen (Preparing of IAC-columns; an english version available).

7.3 SOP ARO/381, User Manual GC-MS Engine Hewlett Packard
For additional general laboratory procedures see: ARO/MIS

7.4 SOP ARO/374, Preparation and validation of reference standards.

8. Conclusions

The analysis of estradiol in serum is based upon an extrelut®-extraction with ethyl acetate, immuno affinity chromatography or solid phase extraction, followed by a derivatization with a mixture of HFBA/Acetone (1:4). Estradiol-di-HFB-derivatives are injected on GC-MS (EI-SIM-mode) and m/z 664 (17ß-E2) and 667 (internal standard = 17ß-E2-d3) are monitored. This method gives good results. The limit of detection is 20 ng/l or ng/kg (S/N > 3 , based on the detection of the most abundant diagnostic ion). The limit of determination is 40 ng/l or ng/kg (S/N > 6). For a single analysis 10 ml serum is used.

9. LOGFORM GC-MS ENGINE

Research plan	**Project title:** **Subtitle:**

Date		Gas chromatography		Analyte	Retention time
Preparation		Column	BPX-5 (non polar)	17ß-E2	10.51 min
in labjournal		Septum purge	ml He / min	17ß-E2-d3	10.50 min
Derivatization	25 µl HFBA / Acetone (1:4) 1 hour, 60? C	Total flow (split vent)	30 ml He / min		min
		He flow	1.00 ml He / min		min
Operator		Length / i.D.	50 m / 0.22 µm		min

~~**SCAN**~~ **/ SIM**	HED on / ~~off~~	M.S.-Mode:	E.I. ~~D.I.P.~~ ~~P.C.I.~~ ~~N.C.I.~~	Tune report file:	EIHEDTUN.U

Scan-mode:		Sim-mode:	Start (min)	Stop (min)	m/z	m/z	m/z	m/z	m/z
Time window	- min	Time window group 1	10.0	12.0	664	667			
Mass range	-	Time window group 2							
Scans per sec.		Time window group 3							
Scan threshold		Dwell time	60 ms						

GC / ~~DIP~~ -parameters

Start temperature	100 ? C	Total temp. program	12.0 min	Inlet A Pressure Program	
During	1 min	Injector temperature A	? C 250	Initial pressure	50 psi
Rise time	20 ? C / min	Detector temperature B	? C 300	Initial time	1 min.
End temperature	300 ? C	µl injection : solution :	5 µl inj. / 25 µl isooctane	Rate	99 psi / min
During	1.0 min	MS Source :	250 ? C	Final press.	27.4 psi
		MS Quad :	120 ? C	Final time:	0 min

Method file name	.M
Macro name : **deuser.mac**	C:\HPCHEM\MSEXE\ .MAC
Sequence : **sample table name**	.S
Data files : **beginning datafile**	.D
: **last datafile**	.D

10. Abbreviations

ARO	Laboratorium voor analytisch residu onderzoek ; Laboratory for Residue Analysis
BSTFA	N,O-Bis(trimethylsilyl)trifluoracetamide
CAS	Chemical Abstracts Service
CB	Cardbox (database system)
CRL	Community Reference Laboratory
DIP	Direct Insertion Probe
17ß-E2	17ß-Estradiol
17ß-E2-d3	17ß-Estradiol-d3 (deuterated)
EU	European Union
EI	Electron Impact
EtAc	Ethyl Acetate
GC-MS	Gas Chromatography-Mass Spectrometry
hXXX	Head of XXX
HED	High Energy Dynode
HFBA	Heptafluorobutyric acid anhydride
IAC	Immuno Affinity Chromatography
IgG	Immunoglobulin G
I.S.	Internal Standard
LC	Liquid Chromatography
m/z	mass/charge
MIS	Management Information System
MW	Molecular Weight
NCI	Negative Chemical Ionization
PCI	Positive Chemical Ionization
ppt	Parts per trillion (pg / ml)
RIA	Radio Immuno Assay
RIVM	Rijksinstituut voor Volksgezondheid en Milieu; National Institute of Public Health and the Environment.
RM	Reference Material
SIM	Selected Ion Monitoring
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
17ß-T	17ß-Testosterone
17ß-T-d2	17ß-Testosterone-d2
TM(C)S	Trimethyl(chlor)silan
UV	Ultra Violet