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1. INTRODUCTION

1.1 Summary
This SOP describes a multi-residue procedure using automatic sample preparation (solid phase extraction (SPE), coupled column-High Pressure Liquid Chromatography (HPLC) and final determination with Gaschromatography with a Mass selective detector (GC-MS). The method is suitable for screening and confirmation of 17 anabolic compounds. After SPE the extract is injected onto a coupled-column HPLC-system for extra clean-up. The extraction of samples of urine is performed automatically with an Automatic Sample Preparation System (Aspec® System) equipped with disposable SPE columns in combination with coupled column reversed phase liquid chromatography.

1.2 Field of application
This method is developed for analysis of anabolic agents in bovine, ovine- and porcine- urine samples. The following 17 compounds are tested for:
- 17α-testosterone (17α-T), 17β-testosterone (17β-T), 17α-19-nortestosterone (17α-NT), 17β-19-nortestosterone (17β-NT), methyl-testosterone (MT), 17α-estradiol (17α-E2), 17β-estradiol (17β-E2), 17α-ethynylestradiol (17α-EE2), diethylstilbestrol (DES), 4-chloro-4-androst-3,17 dione (CLAD), 1-dehydrotestosterone (17α- and 17β-Bolde-none), 1-dehydromethyltestosterone (Methylboldenone), α-Zearalenol, β-Zearalenol, Zeranol and Taleranol.

The method is suitable for screening (chapter 5) and confirmation (chapter 6).

2. REFERENCES

3. H.J. van Rossum et al, Multi residue analysis anabolic agents, SOP ARO/113, revision 4, 21 januari 1997, RIVM.
3. PERFORMANCE CHARACTERISTICS

- The limit of detection for the analytes in sample of bovine- and porcine urine ranges from 1 - 0.1 ppb, for samples of ovine urine equals 2 ppb.
- Confirmation of the identity is possible from 2 ppb.
- The accuracy of the method is for most compounds between 90 and 120%.


4. MATERIALS

4.1 Standards

The use of internal standards is important for the quality control of analytical procedures:
- for correction of the analytical recovery in quantitative analyses
- for control of false negative results in qualitative methods.

Relevant standards are listed in Table 1.

A number of deuterated internal standards is available (Table 2). The standards used for identification and calibration are registered (ARO-MIS Cardbox®). Minimum available data are:
a data-sheet of the source preparation which includes, chemical name, used synonym, CAS-number, molecular weight and a mass spectrum of the actual preparation from which the correct identity can be checked. From these standard preparations 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 (identity). The stock solutions are stored in the dark at -20°C for a maximum period of 5 years. Working solutions are prepared by 10-fold dilution of the stock solutions with ethanol. These solutions are stored in the dark at 4°C (range 1-10°C) for a maximum period of 6 months.

If the corresponding internal standard is not available the compound which elutes closes to the compound of interest should be used as internal standard.
Table 1: Analytes.

<table>
<thead>
<tr>
<th>analyte</th>
<th>abr.</th>
<th>CAS #</th>
<th>formula</th>
<th>Mwt</th>
</tr>
</thead>
<tbody>
<tr>
<td>17α-19-Nortestosterone</td>
<td>1α-NT</td>
<td>4409-34-1</td>
<td>C_{18}H_{26}O_{2}</td>
<td>274.3</td>
</tr>
<tr>
<td>17β-19-Nortestosterone</td>
<td>17β-NT</td>
<td>434-22-0</td>
<td>C_{18}H_{26}O_{2}</td>
<td>274.3</td>
</tr>
<tr>
<td>17α-Testosterone</td>
<td>17α-T</td>
<td>481-30-1</td>
<td>C_{18}H_{26}O_{2}</td>
<td>288.4</td>
</tr>
<tr>
<td>17β-Testosterone</td>
<td>17β-T</td>
<td>58-22-0</td>
<td>C_{18}H_{26}O_{2}</td>
<td>288.4</td>
</tr>
<tr>
<td>17α-Methyltestosterone</td>
<td>MT</td>
<td>58-18-4</td>
<td>C_{20}H_{30}O_{2}</td>
<td>302.4</td>
</tr>
<tr>
<td>17α-1-dehydrotestosterone</td>
<td>α-Bol</td>
<td></td>
<td>C_{18}H_{26}O_{2}</td>
<td>286.4</td>
</tr>
<tr>
<td>17β-1-dehydrotestosterone</td>
<td>β-Bol</td>
<td>846-48-0</td>
<td>C_{18}H_{26}O_{2}</td>
<td>286.4</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>17α-E2</td>
<td>57-91-0</td>
<td>C_{18}H_{24}O_{2}</td>
<td>272.2</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>17β-E2</td>
<td>50-28-2</td>
<td>C_{18}H_{24}O_{2}</td>
<td>272.2</td>
</tr>
<tr>
<td>17α-Ethynyl estradiol</td>
<td>17α-EE2</td>
<td>57-63-6</td>
<td>C_{26}H_{22}O_{2}</td>
<td>296.4</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>DES</td>
<td>56-53-1</td>
<td>C_{18}H_{20}O_{2}</td>
<td>268.4</td>
</tr>
<tr>
<td>4-Chloro-4-androst-3,17 dione</td>
<td>CLAD</td>
<td></td>
<td>C_{19}H_{27}ClO_{3}</td>
<td>320.0</td>
</tr>
<tr>
<td>1-dehydroxymethyltestosterone</td>
<td>Mbol</td>
<td>72-63-9</td>
<td>C_{20}H_{28}O_{2}</td>
<td>300.4</td>
</tr>
<tr>
<td>Zeranol</td>
<td>ZER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taleranol</td>
<td>TAL</td>
<td>42422-68-4</td>
<td>C_{18}H_{26}O_{5}</td>
<td>322.4</td>
</tr>
<tr>
<td>α-Zearalenol</td>
<td>α-ZER</td>
<td>36455-72-8</td>
<td>C_{18}H_{22}O_{5}</td>
<td>320.0</td>
</tr>
<tr>
<td>β-Zearalenol</td>
<td>β-ZER</td>
<td>71030-11-0</td>
<td>C_{18}H_{22}O_{5}</td>
<td>320.0</td>
</tr>
</tbody>
</table>

Table 2: Isotope enriched internal standards.

<table>
<thead>
<tr>
<th>Internal standard</th>
<th>abr.</th>
<th>RIVM/ARO sample no.</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethylstilbestrol-d6</td>
<td>DES-d6</td>
<td>H145661</td>
<td>RIVM</td>
</tr>
<tr>
<td>Zeranol-d4</td>
<td>ZER-d4</td>
<td>87M1553</td>
<td>RIVM</td>
</tr>
<tr>
<td>Taleranol-d4</td>
<td>TAL-d4</td>
<td>87M1553</td>
<td>RIVM</td>
</tr>
<tr>
<td>α-Zearalenol-d4</td>
<td>α-Zer-d4</td>
<td>95M1936</td>
<td>RIVM</td>
</tr>
<tr>
<td>β-Zearalenol-d4</td>
<td>β-Zer-d4</td>
<td>95M1937</td>
<td>RIVM</td>
</tr>
<tr>
<td>17β-19-Nortestosterone-d3</td>
<td>17β-NT-d3</td>
<td>87M1056</td>
<td>RIVM</td>
</tr>
<tr>
<td>Methyltestosterone-d3</td>
<td>MT-d3</td>
<td>H146525</td>
<td>RIVM</td>
</tr>
<tr>
<td>17β-Estradiol-d3</td>
<td>17β-E2-d3</td>
<td>89M1691</td>
<td>MSD md-2325</td>
</tr>
<tr>
<td>3-Chlorotestosterone-d3</td>
<td>Chl.T.-d3</td>
<td>94M0585</td>
<td>Liège (B)</td>
</tr>
<tr>
<td>1-Dehydroxymethyltestosterone-d3</td>
<td>MT-d3</td>
<td>95M0465</td>
<td>RIVM</td>
</tr>
<tr>
<td>17β-Testosterone-d2</td>
<td>17β-T-d2</td>
<td>89M1692</td>
<td>MSD MD-2962</td>
</tr>
<tr>
<td>17α-Ethynylestradiol-d4</td>
<td>17α-EE2-d4</td>
<td>94M5558</td>
<td>C/D/N isotopes D4319</td>
</tr>
</tbody>
</table>
4.2 Chemicals
Reference to any product or company is for purpose of identification and information and does not imply any recommendation of any company by the RIVM.

4.2.1 SPE extraction column, 6 ml disposable octadecyl (C18), Bakerbond or Alltech
4.2.2 SPE extraction column, 6 ml disposable amino (NH2), Bakerbond or Alltech
4.2.3 Two HPLC columns LiChrospher 100 EcoCart 125-3 RP-18 (5 mm) (Merck art. no. 1.50159)
4.2.4 Methanol (Baker Analyzed art. no. 8045)
4.2.5 Ethanol (Baker Analyzed, art. no. 8006)
4.2.6 Acetone (Baker Analyzed, art. no. 8001)
4.2.7 Iso-octane (Baker Analyzed art. no. 8715)
4.2.8 Acetone (Romil, high dry, anhydrous solvent art. no. E450433)
4.2.9 Heptafluorobutyric Acid Anhydride (Pierce, art. no. 63164)
4.2.10 N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey Nagel, art.no.70127)
4.2.11 Ammonium iodide (Fluka, art. no. 09874)
4.2.12 Dithioerythriol (Sigma, art. no. D-9779)
4.2.13 Acetic acid (Merck, art. no. 63)
4.2.14 Sodium acetate (Merck, art. no. 6268)
4.2.15 ß-glucuronidase/sulfatase (suc d'Helix Pomatia containing 100.000 units ß-glucuronidase and 100.000 units sulfatase per ml. Industrie Biologique (France, code IBR 213473)
4.2.16 Acetate buffer, 2 mol/l, pH 5.2 Dissolve 25.2 g of acetic acid (5.14) and 129.5 g of sodium acetate (5.15) in 800 ml water. Adjust the pH, with a pH meter (8.9), at 5.2±0.1 and add water to a final volume of 1000 ml.
4.2.17 HPLC solvent: methanol:water (7:3) (V/V). Add to 700 ml of methanol (5.4) water to a final volume of 1000 ml.

4.3 Samples.
Samples of urine 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 to be within 2 days.

4.4 Instrumentation.
4.4.1 ASPEC® system, type ASPEC XL (see fig. 2) (ASPEC®, Gilson), consists of three components, a model 401C dilutor, a sample processor and a set of racks and accessories to handle 6 ml SPE columns. The system is equipped with an injection-valve (loop-volume 10 ml and 2 ml) for on-line injection of the last eluate into the HPLC system. The system is programmed using the Gilson Sample Manager 721 software. A "MUST" - system (MUST-IET:Multi-port Stream-switch, Spark Separation) couples two analytical columns to the ASPEC® system and the HPLC-system (figure 1).
4.4.2 HPLC-system.(Pharmacia-LKB)
The HPLC-system consists of two LKB 2150 HPLC-pumps, a LC-Controller and a Spectra Focus absorbance detector (UV).
4.4.3 Collecting tubes for the ASPEC system, Gilson type B54728-4.
4.4.4 Spectra Focus absorbance detector (UV) (Spectra Physics). The UV-detector is necessary for recording of the correct time for column-switching.
4.4.5 Derivatization vials, Screw Top Vial with Silicone/PTFE Septa (Omnilabo, Cat.No.154920).

4.4.6 Injection vials, Wide Mouth Crimp (Alltech, no.98213), with micro inserts (100 µl) (Alltech EK-1022.395).

4.5 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 (type 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.

5. SCREENING METHOD

5.1 Introduction
This method describes a multi residue method using automated SPE techniques for the analysis of 17 anabolic compounds in urine of farm animals.

5.2 Summary
Schematic set-up of the coupled-column HPLC system for the analysis of anabolic agents in samples of urine. 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.

![Figure 1. Schematic overview of system.](image-url)
The procedure involves an automated SPE extraction and reversed phase coupled column HPLC procedure for detection (and when possible confirmation) of individual Anabolic Agents (AnAg) in cattle urine. The automated extraction includes 3 fundamental steps: column preconditioning, column washing and elution of the analytes. The coupled-column HPLC system consists of two analytical columns, coupled via a switching valve. The eluent is injected onto column-1 with the valve in the waste position. After this injection the valve is switched to the elute position (Figure 1) and the analyses will continue on both analytical columns of a HPLC system. After collection, evaporation and derivatization of the extract, the analytes are detected with GC-MS.

5.3 Analytical procedures.

5.3.1 Sample pre-treatment.
Urine, 10 ml, is spiked with an internal standard mix (17ß-NTd3, MTd3, ZER-d4, TAL-d4, α-Zer-d4, β-Zer-d4, 17ß-T-d2, 17ß-TB-d2, 17ß-EE2d3, 17α-EE2d4, MBoI-d4, DESd6 and Chl.Td3) (50 ng) and 40 µl of Suc Helix Pomatia, is diluted with 1 ml of 2M acetate buffer. Vortex the mixture for 30 sec., control the pH for being 5.2 (eventually adjust the pH with acetic acid at 5.2) and hydrolysed overnight at 37°C. After hydrolysis the mixture is centrifuged for 2 minutes at 3600 rpm.

5.3.2 Analytical follow-up ASPEC®

![Figure 2. The ASPEC® system.](image-url)
The ASPEC® system (Figure 2) is equipped with 5 racks on the tray platform. First there is a solvent-rack with possibility for 4 different solvents. Second there is a sample-rack for 14 samples and next to it 3 racks for SPE columns with special racks for collection tubes. The pre-treated samples are placed in the sample-rack on the tray platform of the ASPEC® system. In the solvent rack of the ASPEC® system are placed methanol, a mixture of methanol and milli-Q-water (40:60) and acetone. The 6 ml C18 (SPE) disposable columns and 6 ml amino (NH2) disposable columns are fitted with polypropylene caps and installed in the SPE column racks. The collection tubes are placed in the collection racks of the ASPEC® system.

The system is programmed using the Gilson Sample Manager 721 software. The program of the ASPEC® system can directly be made from the Gilson control panel. Appendix I, contains a listing of the program used. All programs are stored electronically and are run from a 3.5’ disk.

The extractions are performed automatically as follows:
- The SPE column racks are reset to the extraction position by the needle of the 401 dilutor (Figure 3-3a).
- The (6 ml) C18 column is preconditio-
- The pre-treated sample (9.9 ml) is
- The C18 column is washed by passing 5 ml of milli-Q water and
- The eluens (3 ml 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.
- The amino column is preconditioned by passing 5 ml of methanol/milli-Q water (40:60) through the column at a flow rate of 6 ml/min. After that, the SPE column rack is moved to the collection position.
- The collected eluate (3 ml 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 push residual solvent through the column. The eluate of this extraction is collected in a collection tube, diluted with 7.0 ml of H2O and mixed by 5 ml of air.

Figure 3.
3a= extraction position
3b= collection position
- The total eluate (10 ml) is then injected onto column-1 of the HPLC system. For the first 20 minutes following injection, column-1 effluent is sent directly to the waste with H₂O to remove the acetone solvent. Column-1 is then coupled, via a switching valve, to column-2 and analytes eluting with a mixture of methanol water (70:30) from column-2, at a flow rate of 0.6 ml/min and the eluate is collected by using a fraction collector.

- The first 4 minutes after column-switching effluent is sent to waste to prevent it from interfering with early eluting analytes. After that, a fraction of 15 minutes is collected (about 9 ml).

After this cycle, the ASPEC® system continues with the extraction of the next sample until all the samples are processed.

5.3.3 Derivatization.

The eluates are evaporated at 50°C under a stream of nitrogen until dryness and dissolved in methanol (0.5 ml). These extracts are separated in two parts of 250 µl and transferred into two different derivatization-vials and evaporated at 50°C under a stream of nitrogen until dry. The dry residue in the first vial is derivatized with 25 µl of heptafluorobutyric acid anhydride/acetone p.a. (1:4) for the drugs 17α-T and 17β-T, 17α and 17β-NT, α- and β-Bol, MBol, 17α- and 17β-E2, 17α-EE2, MBol-d4, 17β-N'd3, MT-d3, 17β-T-d2, β-E-d2, 17α -EE4. The residue in the other vial is derivatized with 20 µl of a mixture N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)-ammoniumiodide-dithioerythritol (1000:2:4, v/w/w) for the drugs DES, DES-d6, CLAD, 3-Chl.T.-d3, α and β-Zer, ZER, TAL, ZER-d4, TAL-d4. The derivatization-vials are placed in a heater at 60°C, after 1 hour evaporated at 50°C under a stream of nitrogen until dry and dissolved in iso-octane (25 µl) and transferred into an injection-vial with micro insert. The 7673 automatic sampler injects 3 µl of these extracts in the GC-MS for analysis.

5.3.4 GC-MS condition.

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 at 30°C/min. to 300°C and held at this final temperature for 5 min. Injector temperature is maintained at 250°C and detector temperature is at 250°C for the MS-source and at 120°C for the MS-quadrupool. For quantitative analysis, we used a selected ion monitoring (SIM) program, monitoring ions 664 for 17α and 17β-E2, 667 for 17β-E3, 666 for 17α and 17β-NT, 669 for 17β-N'T-d3, 678 for α and β-Bol, 680 for 17α and 17β-T, 682 for 17β-Td2, 465 for MT, 468 for MT-d3, 478 for M-Bol, 481 for M-Bol-d3, 474 for 17α-EE2, 478 for 17α-EE2-d4, 412 for DES, 418 for DES-d6, 464 for CLAD, 469 for 3-Chl.T.d3, 446 for α-Zer and β-Zer, 433 for ZER and TAL, 437 for ZER-d4 and TAL-d4. In Table 4 the diagnostic ions for each of the compounds are summarized. The instrument is operated in the electron-impact ionization (EI) mode. The injection port is set in the splitless mode by 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 oven-temperature program.
Table 4. Diagnostic ions.

<table>
<thead>
<tr>
<th>Anabolic agents</th>
<th>Abr.</th>
<th>Abr. Int. standard</th>
<th>int. st. ions</th>
<th>Diag. ions*</th>
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</thead>
<tbody>
<tr>
<td>CLAD</td>
<td>CLAD</td>
<td>Chl.T.-d3</td>
<td>469</td>
<td>464, 392, 357, 221</td>
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<tr>
<td>DES</td>
<td>DES</td>
<td>DES-d6</td>
<td>418</td>
<td>412, 397, 383, 413</td>
</tr>
<tr>
<td>α-Zearalanol</td>
<td>ZER</td>
<td>ZER-d4</td>
<td>437</td>
<td>538, 433, 335, 307</td>
</tr>
<tr>
<td>β-Zearalanol</td>
<td>TAL</td>
<td>TAL-d4</td>
<td>437</td>
<td>538, 433, 335, 307</td>
</tr>
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<td>α-Zearalenol</td>
<td>α-Zer</td>
<td>α-Zer-d4</td>
<td>450</td>
<td>536, 446, 333, 305</td>
</tr>
<tr>
<td>β-Zearalenol</td>
<td>β-Zer</td>
<td>β-Zer-d4</td>
<td>450</td>
<td>536, 446, 333, 305</td>
</tr>
<tr>
<td>α-Estradiol</td>
<td>17α-E2</td>
<td>17β-E2-d3</td>
<td>667</td>
<td>664, 451, 409, 356</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>17β-E2</td>
<td>17β-E2-d3</td>
<td>667</td>
<td>664, 451, 409, 356</td>
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<td>α-NT</td>
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<td>17β-NT-d3</td>
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<td>666, 453, 306, 133</td>
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<td>678, 464, 369, 169</td>
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<td>669</td>
<td>678, 464, 369, 169</td>
</tr>
<tr>
<td>M-Boldenon</td>
<td>MBol</td>
<td>MBol-d3</td>
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<td>478, 367, 435, 463</td>
</tr>
<tr>
<td>MT</td>
<td>MT</td>
<td>MT-d3</td>
<td>468</td>
<td>480, 465, 369, 355</td>
</tr>
<tr>
<td>α-T</td>
<td>17αT</td>
<td>17β-T-d2</td>
<td>682</td>
<td>680, 467, 355, 320</td>
</tr>
<tr>
<td>β-T</td>
<td>17β-T</td>
<td>17β-T-d2</td>
<td>682</td>
<td>680, 467, 355, 320</td>
</tr>
<tr>
<td>EE2</td>
<td>17α-EE2</td>
<td>17α-EE2-d4</td>
<td>478</td>
<td>474, 459, 446, 353</td>
</tr>
</tbody>
</table>

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

5.4 Quality control.

5.4.1 Checking procedure, moment of column-switching.

From a standard solution of β-NT containing 1 ng per ml, 100 µl is diluted with a mixture of acetone and water (3:7) until a total volume of 10 ml. This solution of 10 ml is directly injected into the injection valve of the HPLC-system, onto column-1 with the valve V-2 of the "Must" system (figure 1) in the "waste" position. The injection time is at least 17 minutes because the flow of the HPLC pump is 0.6 ml per minute. After this injection, the valve V-2 is switched to the "elute" position and both analytical columns is eluted with a mixture of methanol and water (70:30). In this case, the UV-detector is used at 254 nm (maximum wavelength for β-NT).

5.4.2 Calibration curves.

A calibration curve is constructed by analyzing different concentrations of standard, at least 5 concentrations. The calibration curves are prepared by a standard mix of 17 anabolic agents (Table 1), with a concentration of 0.5 ng/ml (each compound) and an internal standard mix of 13 deuterated compounds (Table 2) with a concentration of 0.5 ng/ml (each compound). Before injection into a HPLC system, 100 µl (50 ng) of the internal standard mix and an amount of the standard mix (ranging 25 to 125 µl) is mixed in a tube. This solution is diluted with a mixture of acetone and water (3:7) to a final volume of 10 ml. The total solution (10 ml) is injected onto column-1 of the HPLC system. For the first 20 minutes following injection, column-1 effluent is sent directly to waste to remove the acetone solvent. Column-1 is coupled, via a switching valve, to column-2 and analytes eluting from column-2 are collected by using a fraction collector.
After evaporation and derivatization, the analytes are quantitated by GC-MS. Peak area ratios of 17α-NT, 17β-NT, α-Bol, β-Bol are measured to 17β-NT deuterated (17β-NT-d3); M Bol to MBol deuterated (MBol-d3); MT to MT deuterated (MTd3); DES to DES deuterated (DES-d6), CLAD to 3-Chl.T. deuterated (3-Chl.T-d3); 17α-T, 17β-T to 17β-T deuterated (17β-Td2); 17α-E2, 17β-E2 to 17β-E2 deuterated (17E2-d3); α-Zer, β-Zer, to α-Zer and β-Zer deuterated (α-Zer-d4 and β-Zer-d4), ZER, TAL to ZER and TAL deuterated (ZER-d4 and TAL-d4), 17α-EE2 to 17α-EE2 deuterated (17α-EE2-d4). The calibration curves are generated by linear regression. The regression lines are used to calculate the extraction yields of the analytes. For calculation Calwer® program is used (see ref. 10 and Appendix III).

6. SEPARATION AND CONFIRMATION METHOD

6.1 Introduction.

HPLC with UV-detection is a rapid but not so selective technique for the identification of anabolics. This technique is used to separate and collect the anabolic compounds after which final determination with GC-MS is performed. The coupled-column HPLC system in this method, gives cleaner extracts and less background, when compared to SPE clean-up only, on the GC-MS. With this method, in most of the cases, it is possible to confirm the presence of anabolics on four ions, according to the EC-criteria (ref. 9).

Schematic set-up of the coupled-column HPLC system for the 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).

Figure 4. Elution position.
6.2 Analytical procedures.

6.2.1 Sample pretreatment.

The overall scheme of the analytical procedure is given in Figure 5. Urine 10 ml; added 40 µl Suc d’Helix Pomatia and 1 ml of 2M acetate buffer; 30 sec. vortexing the mixture and (pH at 5.2 see 5.3.1) hydrolysed over night at 37°C. After hydrolysis the mixture is centrifuged for 2 minutes at 3600 rpm.

6.2.2 ASPEC® clean-up.

The solid-phase extraction is performed automatically by a Gilson System equipped with disposable Bakerbond SPE columns C18 and NH₂ (amino) in combination with coupled-column reversed phase liquid chromatography. The procedure is as follows:
- The C18 column is preconditioned with 5 ml of methanol and 5 ml of milli-Q water. The sample is loaded onto the column. The C18 column is washed with 5 ml of milli-Q water and a mixture of 4 ml methanol/milli-Q water (40:60) and the analytes are eluted with 3 ml of acetone.
- The NH₂- column is preconditioned with a mixture of 5 ml of methanol/milli-Q water (40:60) and the collected eluate (3 ml of acetone) from C18 is dispensed onto the amino column. This eluate is collected, evaporated (at 50°C under a gently stream of nitrogen) till approximately 1 ml and diluted with 1 ml of water.
- Coupled column HPLC.

The total eluate (2 ml) is injected onto 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 that column-1 is coupled, via a switching valve, to column-2 and 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’s 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 (see Table 5).

Figure 6 shows a chromatogram of α and β Zearalanol, α and β Zearalenol and DES eluting under the described conditions, obtained with UV detection. The technique with gradient elution results in good separation. Different fractions can be collected each fraction containing a number of anabolics.

Figure 7 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) elution the columns, fraction collection, reconditioning the columns.
Table 5. Analytes retention times on HPLC and collection fractions.

<table>
<thead>
<tr>
<th>Anabolic agents</th>
<th>Rtime *(min)</th>
<th>Fract. 1</th>
<th>Fract. 2</th>
<th>Fract. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLAD</td>
<td>33.5</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td>35.4</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>ZER</td>
<td>29.7</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>TAL</td>
<td>20.7</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>α-Zer</td>
<td>32.3</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>β-Zer</td>
<td>23.8</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17α-E2</td>
<td>32.1</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>17β-E2</td>
<td>32.1</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17β-NT</td>
<td>28.4</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>17α-NT</td>
<td>32.4</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17β-Bol</td>
<td>24.9</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>17α-Bol</td>
<td>30.3</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>MBol</td>
<td>29.2</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>MT</td>
<td>37.4</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17β-T</td>
<td>32.5</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17α-T</td>
<td>37.3</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>EE2</td>
<td>32.1</td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

(* = Retention time liquid chromatogram). (+ = collected in this fraction).

Figure 6. Chromatogram of standard solution containing (1) Taleranol, (2) β-Zearalenol, (3) Zeranol, (4) α-Zearalenol and (5) DES.
The analytes which included this procedure and the retention time of each compound are listed in table 2. It can be seen that most anabolics are eluted in the range between 20 - 37 minutes.

6.2.3 Derivatization.

The fraction containing the analyte to be confirmed is evaporated at 50°C under a stream of nitrogen until dryness and dissolved in methanol (0.5 ml). The extract is transferred into a derivatization-vial and evaporated at 50°C under a stream of nitrogen until dryness. The dry residue is derivatized with 25 µl of a of heptafluoro butyric acid anhydride/ace-tone p.a. (1:4) for the drugs α- and β-T, α - and β-NT, α - and β-Bol, MBol, 17α - and 17β-E2, 17α-EE2. For the drugs DES, CLAD, α and β-Zer, ZER, TAL, the residue is derivatized with 20 µl of a mixture N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA)-ammonium iodide-dithioerythritol (1000:2:4, v/v/w). The derivatization-vials are placed in a heater at 60°C for 1 hour. Evaporate at 50°C under a stream of nitrogen until dry and dissolved in iso-octane (25 µl) and transferred into an injection-vial with micro insert. The 7673 automatic sampler injects 3 µl of these extracts into the GC-MS and measures these extracts, in the SIM-mode (for ions see Table 4). For the analyte to be confirmed at least three standard solutions, are processed as described under 5.3. The first solution contains approximately 50%, the second 100% and the third 200% of the estimated amount available from the sample.

6.2.4 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 micron 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. Injector temperature is maintained at 250°C and detector temperature is 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.

Figure 7. Gradient conditions and the collection windows.
6.3 Evaluation of the results.

For identification (confirmation) according to the criteria laid down by the European Commission, at least 4 diagnostic ions should be monitored. The ratios are calculated by dividing the response of a (diagnostic) ion and the target ion (most abundant).

Table 6. The ratios for each compound calculated by injection of a standard analysed with the whole method.

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

The ratios in Table 6 are obtained by injecting 100 ng of a standard, after cleaning and derivatization with the method described and are included as an indication of the values to be expected. However, these ratios are strongly influenced by experimented conditions at the moment of detection. The ratios for the sample have to fit within the average value obtained for the standards ± 10%.
Appendix 1.

ASPEC® program Screening.
Gilson 731 ASPEC Software V1.00

CONFIGURATION Sampler
Model : ASPECi
Arm (mm) : 183
Rinsing station depth (mm) : 80
Nb of rinsing stations : 1
Injection loop volume (µl) : 1990
Injection flush volume (µl) : 1990
Transfer ports : NO
ID number : 10

CONFIGURATION Dilutor
Syringe volume (µl) : 10000
Transfer tubing (µl) : 10000
ID number : 0

CONFIGURATION Default parameters
Air gap volume (µl) : 25
Air gap asp. flow (ml/min) : 5
Reservoir asp. flow (ml/min) : 5
Prime disp. flow (ml/min) : 5
Rinsing volume (µl) : 1000
Disp. rinse flow (ml/min) : 5
Immersion rinse (mm) : 80
Level detection sensitivity : 5
Analog input range (mV) : 10

TRAY racks
Rack | 61 | 24 | 106 | 106 | 101 | (= rack codes)
Pattern | I | Z | Z | Z | Z | (= moving arm)

TRAY zones
<table>
<thead>
<tr>
<th>Zone</th>
<th>first</th>
<th>last</th>
<th>l.det.</th>
<th>vial i.d.</th>
<th>total vol</th>
<th>depth</th>
<th>height</th>
</tr>
</thead>
<tbody>
<tr>
<td>solvent B¹</td>
<td>2</td>
<td>2</td>
<td>OFF</td>
<td>53 mm 250000µl</td>
<td>-</td>
<td>0 mm</td>
<td></td>
</tr>
<tr>
<td>solvent A²</td>
<td>1</td>
<td>1</td>
<td>OFF</td>
<td>53 mm 250000µl</td>
<td>-</td>
<td>0 mm</td>
<td></td>
</tr>
<tr>
<td>solvent C³</td>
<td>3</td>
<td>3</td>
<td>OFF</td>
<td>53 mm 250000µl</td>
<td>-</td>
<td>0 mm</td>
<td></td>
</tr>
<tr>
<td>sample</td>
<td>5</td>
<td>18</td>
<td>ON</td>
<td>25.2 mm</td>
<td>-</td>
<td>2 mm 0 mm</td>
<td></td>
</tr>
<tr>
<td>dec A</td>
<td>19</td>
<td>33</td>
<td>OFF</td>
<td>9.5mm</td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
<tr>
<td>collectA</td>
<td>19</td>
<td>33</td>
<td>OFF</td>
<td>9.5mm</td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
<tr>
<td>dec B</td>
<td>34</td>
<td>48</td>
<td></td>
<td></td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
<tr>
<td>collectB</td>
<td>34</td>
<td>48</td>
<td>OFF</td>
<td>9.5mm</td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
<tr>
<td>dec C</td>
<td>49</td>
<td>84</td>
<td></td>
<td></td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
<tr>
<td>collectC</td>
<td>49</td>
<td>84</td>
<td>OFF</td>
<td>9.5mm</td>
<td>-</td>
<td>- 0 mm</td>
<td></td>
</tr>
</tbody>
</table>

¹ SolventB = methanol/water (40:60)
² SolventA = methanol
³ SolventC = acetone
1-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number: X (standby fraction-collector)
State: CLOSE

2-Begin Loop

3-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number: X (Wash-position "Must"-valve)
State: CLOSE

4-CONDITION
--------- Dec zone ---------
Zone name: decA (C18 - 6 ml column)
Press. eq. time (min): 0.5
--------- Solvent zone ---------
Zone name: solventA (methanol)
Volume (ml): 5
Air push volume (ml): 2
Disp. flow (ml/min): 6
Asp. flow (ml/min): 6

5-CONDITION
--------- Dec zone ---------
Zone name: decA (C18 - 6 ml column)
Press. eq. time (min): 0.5
--------- Solvent zone ---------
Zone name: RESERVOIR (water)
Volume (ml): 5
Air push volume (ml): 2
Disp. flow (ml/min): 6

6-LOAD
--------- Dec zone ---------
Zone name: decA (C18 - 6 ml column)
Press. eq. time (min): 0.5
--------- Source zone ---------
Zone name: sample
Volume (ml): 9.9
Air push volume (ml): 2
Disp. flow (ml/min): 6
Asp. flow (ml/min): 6

1 Contact number depend of the configuration of the instrument. See the manual of the instrument.
7-WASH

---------- Dec zone ----------
Zone name             : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name             : RESERVOIR (water)
Volume (ml)           : 5
Air push volume (ml) : 2
Disp. flow (ml/min)   : 6

8-WASH

---------- Dec zone ----------
Zone name             : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name             : solventB (methanol/water 40:60)
Volume (ml)           : 4
Air push volume (ml) : 2
Disp. flow (ml/min)   : 6
Asp. flow (ml/min)    : 6

9-ELUTE (C18 column)

-------- Collect zone --------
Zone name             : collectA
Press. eq. time (min) : 0.1

-------- Solvent zone --------
Zone name             : solventC (acetone)
Volume (ml)           : 3
Air push volume (ml) : 2
Disp. flow (ml/min)   : 6
Asp. flow (ml/min)    : 6

10-CONDITION

---------- Dec zone ----------
Zone name             : decB (NH2 - 6ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name             : solventB (methanol/water 40:60)
Volume (ml)           : 5
Air push volume (ml) : 2
Disp. flow (ml/min)   : 6
Asp. flow (ml/min)    : 6
11-LOAD & COLLECT

---------- Dec zone ----------
Zone name : decB (NH2 - 6ml column)
Press. eq. time (min) : 0.5

---------- Source zone ----------
Zone name : collectA (eluate C18)
Volume (ml) : 3
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

12-ADD

---------- Result zone ----------
Zone name : collectB (eluate NH2)

---------- Added Solution ----------
Zone name : RESERVOIR (water)
Volume (ml) : 7
Disp. flow (ml/min) : 6

13-MIX

---------- Source Zone ----------
Zone name : collectB (eluate NH2)

---------- Mixing method ----------
Mixing method : AIR
Air volume (ml) : 5
Disp. flow (ml/min) : 6

14-INJECT (HPLC) (aut. start fraction-collector)

---------- Source zone ----------
Zone name : collectB (eluate NH2)
Asp. flow (ml/min) : 6

---------- Injection mode ----------
Injection Method : TOTAL LOOP
Loop filling coeff. : 5
Disp. flow (ml/min) : 6
Valve flush volume(µl) : 9950

---------- Coordination ----------
Coordination : NO
Chrom. time (min) : 5

---------- Nb of injections ----------
Nb of injections : 1

15-SET ELECTRICAL CONTACT

---------- Set contact ----------
Contact number : X
State : PULSE

1 Contactnumber depend of the configuration of the instrument. See the manual of the instrument.
16-WAIT (wait fraction collector)  
------------- TIME -------------  
Time (min) : 20

17-SET ELECTRICAL CONTACT  
------------- Set contact --------  
Contact number : X\(_{1}\) (switching MUST valve  
State : OPEN

18-WAIT (after 5 min. start collect fract.collector)  
------------- TIME -------------  
Time (min) : 21

19-SET ELECTRICAL CONTACT  
------------- Set contact --------  
Contact number : X\(_{1}\) (stop fraction collector)  
State : CLOSE

20-SET ELECTRICAL CONTACT  
------------- Set contact --------  
Contact number : X\(_{1}\) (change tube fract.collector)  
State : PULSE

21-RINSE INJECTION PORT (HPLC)  
------------- Volume -------------  
Rinsing volume (µl) : 9000  
Disp. flow (ml/min) : 6

22-End Loop

23-SET ELECTRICAL CONTACT  
------------- Set contact --------  
Contact number : X\(_{1}\) (start HPLC pump to standby position))  
State : PULSE

24-SET ELECTRICAL CONTACT  
------------- Set contact --------  
Contact number : X\(_{1}\) (stop fraction collector)  
State : CLOSE

\(^{1}\) Contact number depend of the configuration of the instrument. See the manual of the instrument.
Appendix II.

ASPEC® program Confirmation

Gilson 731 ASPEC Software V1.00

CONFIGURATION Sampler
Model : ASPECi
Arm (mm) : 183
Rinsing station depth (mm) : 80
Nb of rinsing stations : 1
Injection loop volume (µl) : 1990
Injection flush volume (µl) : 1990
Transfer ports : NO
ID number : 10

CONFIGURATION Dilutor
Syringe volume (µl) : 10000
Transfer tubing (µl) : 10000
ID number : 0

CONFIGURATION Default parameters
Air gap volume (µl) : 25
Air gap asp. flow (ml/min) : 5
Reservoir asp. flow (ml/min) : 5
Prime disp. flow (ml/min) : 5
Rinsing volume (µl) : 1000
Disp. rinse flow (ml/min) : 5
Immersion rinse (mm) : 80
Level detection sensitivity : 5
Analog input range (mV) : 10

TRAY racks
Rack | 61 | 24 | 106 | 106 | 101 | (= rack codes)
Pattern | I | Z | Z | Z | Z | (= moving arm)

TRAY zones
zone | first | last | l.det. | vial i.d. | total vol | depth | height
solventB\(^1\) | 2 | 2 | OFF | 53 mm | 250000µl | - | 0 mm
solventA\(^{ii}\) | 1 | 1 | OFF | 53 mm | 250000µl | - | 0 mm
solventC\(^{iii}\) | 3 | 3 | OFF | 53 mm | 250000µl | - | 0 mm
sample | 5 | 18 | ON | 25.2 mm | - | 2 mm | 0 mm
decA | 19 | 33 | | | |
collectA | 19 | 33 | OFF | 9.5 mm | - | - | 0 mm
decB | 34 | 48 | | | |
collectB | 34 | 48 | OFF | 9.5 mm | - | - | 0 mm
decC | 49 | 84 | | | |
collectC | 49 | 84 | OFF | 9.5 mm | - | - | 0 mm

\(^1\) SolventB = methanol/water (40:60)
\(^{ii}\) SolventA = methanol
\(^{iii}\) SolventC = acetone
1-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X₁ (standby fraction-collector)
State : CLOSE

2-Begin Loop

3-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X₁ (Wash-position "Must"-valve)
State : CLOSE

4-CONDITION
--------- Dec zone ---------
Zone name : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5
--------- Solvent zone ---------
Zone name : solventA (methanol)
Volume (ml) : 5
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

5-CONDITION
--------- Dec zone ---------
Zone name : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5
--------- Solvent zone ---------
Zone name : RESERVOIR (water)
Volume (ml) : 5
Air push volume (ml) : 2
Disp. flow (ml/min) : 6

6-LOAD
--------- Dec zone ---------
Zone name : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5
--------- Source zone ---------
Zone name : sample
Volume (ml) : 9.9
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

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1 Contact number depend of the configuration of the instrument. See the manual of the instrument.
7-WASH

--------- Dec zone ---------
Zone name : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name : RESERVOIR (water)
Volume (ml) : 5
Air push volume (ml) : 2
Disp. flow (ml/min) : 6

8-WASH

--------- Dec zone ---------
Zone name : decA (C18 - 6 ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name : solventB (methanol/water 40:60)
Volume (ml) : 4
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

9-ELUTE (C18 column)

-------- Collect zone --------
Zone name : collectA
Press. eq. time (min) : 0.1

-------- Solvent zone --------
Zone name : solventC (acetone)
Volume (ml) : 3
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

10-CONDITION

--------- Dec zone ---------
Zone name : decB (NH2 - 6ml column)
Press. eq. time (min) : 0.5

-------- Solvent zone --------
Zone name : solventB (methanol/water 40:60)
Volume (ml) : 5
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6
11-LOAD & COLLECT

---------- Dec zone ----------
Zone name : decB (NH2 - 6ml column)
Press. eq. time (min) : 0.5

---------- Source zone ----------
Zone name : collectA (eluate C18)
Volume (ml) : 3
Air push volume (ml) : 2
Disp. flow (ml/min) : 6
Asp. flow (ml/min) : 6

12-EVAPORATE

---------- Collect zone ----------
Zone name : collectB (eluate NH2)
time (min) : 20 MIN.

---------- Needle Speed ----------
0.60 (min)
Z - position : 130.00
Purging time (min) : 0.50

13-Dilute

---------- Result zone ----------
Zone name : collectB (eluate NH2)

---------- Added Solution ----------
Zone name : RESERVOIR (water)
Volume (ml) : 1
Disp. flow (ml/min) : 6

14-MIX

---------- Source Zone ----------
Zone name : collectB (eluate NH2)

---------- Mixing method ----------
Mixing method : AIR
Air volume (ml) : 5
Disp. flow (ml/min) : 6
Title: A multi residue method using coupled-column HPLC and GC-MS for determination of anabolic compounds in samples of urine.

15-INJECT (HPLC)( aut. start fraction-collector)

------- Source zone ---------
Zone name : collectB
Asp. flow (ml/min) : 6
------- Injection mode -------
Injection Method : TOTAL LOOP
Loop filling coeff. : 5
Disp. flow (ml/min) : 6
Valve flush volume(µl) : 9950
------- Coordination -------
Coordination : NO
Chrom. time (min) : 5
------- Nb of injections -------
Nb of injections : 1

16-SET ELECTRICAL CONTACT

--------- Set contact ---------
Contact number : X¹ (start HPLC pump)
State : PULSE

17-WAIT (wait fraction collector)

----------- TIME -----------
Time (min) : 4

18-SET ELECTRICAL CONTACT

--------- Set contact ---------
Contact number : X¹ (switching MUST valve)
State : OPEN

19-WAIT (after 5 min. start collect fract.collector)

----------- TIME -----------
Time (min) : 4

20-SET ELECTRICAL CONTACT

--------- Set contact ---------
Contact number : X¹ (stop fraction collector)
State : CLOSE

21-SET ELECTRICAL CONTACT

--------- Set contact ---------
Contact number : X¹ (change tube fract.collector)
State : PULSE

22 - Wait

----------- Time-----------
delay (min) : 28

¹ Contact number depend of the configuration of the instrument. See the manual of the instrument.
23-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X (change tube fract.collector)
State : PULSE

22 - Wait
--------- Time ---------
delay (min) : 8

23-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X (change tube fract.collector)
State : PULSE

24 - Wait
--------- Time ---------
delay (min) : 8

25-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X (change tube fract.collector)
State : PULSE

26-RINSE INJECTION PORT (HPLC)
--------- Volume ---------
Rinsing volume (µl) : 9000
Disp. flow (ml/min) : 6

27-End Loop

28-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X (start HPLC pump to standby position))
State : PULSE

29-SET ELECTRICAL CONTACT
--------- Set contact ---------
Contact number : X (stop fraction collector)
State : CLOSE

1 Contact number depend of the configuration of the instrument. See the manual of the instrument.
Appendix III

Calwer procedures.

The regression lines are used to calculate the concentration of the analytes in the samples. The Linear regression program Calwer\(^{18}\) is used. In Figure 8 an example of the Calwer worksheet is given.

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Figure 8. Calwer worksheet.

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