SOLUTIONS BY LCTech


# Automated Determination of Steroids in Urine of Food-Producing Animals

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# 1 Introduction

In the European Union the use of steroids as growth-promotor for food-producing animals is strictly forbidden. In National Residue Control Plans (Directive 96/23/EC) the analysis of steroids in urine of food-producing animals is prescribed to uncover illegal treatment. Some official laboratories in the European Union have to analyse 1000 samples and more per year. The matrix urine leads to some interference in final measurement using LC-MS/MS or HPLC-HRMS, respectively. Therefore an efficient cleaning of the sample solution using two different SPE columns is needed. Due to the number of samples to be monitored time saving is crucial, and thus an automated sample clean-up is recommendable.

## 1.1 Chemicals

The chemicals of interest are listed in Table1. These substances are often named synthetic steroids, but in the literature it is described, that substances like boldenone and nandrolone can be generated endogenously in boars for example [1].

| Chemicals | Internal Standard |
|---|---|
| Alpha-Boldenone | Beta-Boldenone-D3 |
| Beta-Boldenone | Beta-Boldenone-D3 |
| Alpha-Nortestosterone | Beta-Nortestosterone-D3 |
| Beta-Nortestosterone | Beta-Nortestosterone-D3 |
| Alpha-Trenbolone | Beta-Trenbolone-D3 |
| Beta-Trenbolone | Beta-Trenbolone-D3 |
| Methyl-Testosterone | Methyltestosterone-D3 |
| Stanozolol | Stanozolol-D3 |
| Hydroxystanozolol | Hydroxystanozolol-D3 |
| Ethinylestradiol | Ethinylestradiol-D4 |

Table 1: Steroids of interest

# 2 Method Development

## 2.1 Material

- Formic acid (0.1 %) in acetonitrile (ULC/MS), 0.1 % formic acid in water (ULC/MS) both from Biosolve (the Netherlands)
- Sodium acetate, anhydrous, per analysis from Merck (Germany)
- Ethyl acetate (picograde), methanol (picograde) and acetonitrile (picograde) from Promochem (Germany)
- Glucuronidase/arylsulfatase solution from Sigma (USA)

## 2.2 Sample Material

The urine is centrifuged to separate any particulate matter.

## 2.3 Sample Treatment - Extraction

4 mL of centrifuged urine is diluted with 4 mL of sodium acetate buffer (50 mmol/l, pH 5.2). 20 µL of a glucuronidase/arylsulfatase solution and internal standard solution is added to the sample. The sample is incubated at 37 °C over night. After a centrifugation step, the sample solution is cleaned-up using two different SPE columns.

## 2.4 Solid Phase Extraction (manual)

| Column 1 | CHROMABOND® HR-X, 6 mL, 200 mg |
|---|---|
| Column conditioning | 4 mL methanol<br>4 mL double distilled water |
| Loading step | The sample is completely applied onto the column. |
| Washing step | 4 mL methanol/double distilled water (v/v, 5/95) after a drying step (5 min.)<br>1 mL methanol |
| Elution Step | 3 mL methanol |

Table 2: Conditions of the SPE method for the first column (manual)

| Column 2 | CHROMABOND® $NH_2$, 3 mL, 500 mg |
|---|---|
| Column conditioning | 5 mL methanol<br>5 mL methanol/ethyl acetate (v/v, 40/60) |
| Loading step | The eluate from column 1 is completely applied onto the column. |
| Elution Step | The solution running through the column is collected.<br>After waiting 10 min, vacuum was put to the column for 20 sec. |

Table 3: Conditions of the SPE method for the second column (manual)

## 2.5 Solid Phase Extraction (automated by LCTech)

The manual solid phase extraction can be easily transferred to the robotic system FREESTYLE SPE for automated sample preparation. The software of the system contains pre-defined wizards for every step making the method development fast and easy.

FREE-STYLE™ AUTOMATISIERTE PROBENVORBEREITUNG

LCTech FreeStyle - Report on Methods: DSM Date: 22.03.2016 Time: 16:49:22

Name: Steroids.dsm

| | | | | |
|---|---|---|---|---|
| Column: | LCTech_6ml.col | | Extension cannula: | no |
| Conditioning 1: | ON | | | |
| Volume: | 4 ml | Dispensing Speed: | 6 ml / min | |
| Suction Speed: | 40 ml / min | Waiting time: | 0.1 min | Port : 1 MeOH |
| Conditioning 2: | ON | | | |
| Volume: | 4 ml | Dispensing Speed: | 6 ml / min | |
| Suction Speed: | 50 ml / min | Waiting time: | 0.1 min | Port : 7 H2O |
| Conditioning 3: | OFF | | | |
| Load 1: | ON | | | |
| Volume: | 8 ml | Dispensing Speed: | 2 ml / min | |
| Suction Speed: | 10 ml / min | Input Vial Type: | Type1@15 | |
| Load 1 into Waste | | | | |
| Quantitativ Transfer | 2 x 2ml | | | Port : 7 H2O |
| Drying 0: | OFF | | | |
| Washing 1: | ON | stay on actual position | | |
| Volume: | 1 ml | Dispensing Speed: | 6 ml / min | |
| Suction Speed: | 40 ml / min | Waiting time: | 0.1 min | Port : 8 MeOH/H2O 5/95 |
| Washing 2: | OFF | | | |
| Drying 1: | ON | stay on actual position | | |
| Time: | 1 min | | | |
| Washing 3: | OFF | | | |
| Elution 1: | OFF | | | |
| Drying 2: | OFF | | | |
| Washing 4: | ON | into Waste | | |
| Volume: | 1.4 ml | Dispensing Speed: | 1.5 ml / min | |
| Suction Speed: | 50 ml / min | Waiting time: | 00.1 min | Port : 1 MeOH |
| Drying 3: | OFF | | | |
| Elution 2: | ON | | | |
| Volume: | 2.5 ml | Dispensing Speed: | 2 ml / min | |
| Suction Speed: | 50 ml / min | Waiting time: | 0 min | Port : 1 MeOH |
| | | Vial Type: | Type3@14 | |
| Drying 4: | ON | stay on actual position | | |
| Drying by defined air volume | | Volume: 10 ml | Dispensing Speed: 6 ml / min | |
| Cleaning cycle 1 : | ON | | | |
| Volume: | 1 ml | Dispensing Speed: | 1 ml / min | |
| Suction Speed: | 1 ml / min | | | Port : 1 MeOH |

Figure 1: Method report (part 1) for the dual SPE method (automated)

| | | | | |
|---|---|---|---|---|
| Column SPE_2: | LCTech_3ml.col | | | |
| Extension cannula: | no | | | Same solvent as SPE_1 |
| Conditioning 2.1: | ON | | | |
| Volume: | 5 ml | Dispensing Speed: | 40 ml / min | |
| Suction Speed: | 6 ml / min | Waiting time: | 0.1 min | Port : 1 MeOH |
| Conditioning 2.2: | ON | | | |
| Volume: | 5 ml | Dispensing Speed: | 6 ml / min | |
| Suction Speed: | 50 ml / min | Waiting time: | 0.1 min | Port : 9 40/60 MeOH/Ethylacetate |
| Conditioning 2.3: | OFF | | | |
| Load_2: | ON | From vial with Eluate 2 | | |
| Volume: | 3.2 ml | Dispensing Speed: | 2 ml / min | |
| Suction Speed: | 10 ml / min | Input Vial Type: | Type3@14 | |
| Quantitativ Transfer | 1 x 1 ml | | | Port : 1 MeOH |
| Drying 2.0: | ON | into Waste | | |
| Time: | 0.5 min | | | |
| Washing 2.1: | OFF | | | |
| Washing 2.2: | OFF | | | |
| Drying 2.1: | OFF | | | |
| Washing 2.3: | OFF | | | |
| Elution 2.1: | OFF | | | |
| Drying 2.2: | OFF | | | |
| Washing 2.4: | OFF | | | |
| Drying 2.3: | OFF | | | |
| Elution 2.2: | OFF | | | |
| Drying 2.4: | OFF | | | |
| Washing 4: | OFF | | | |

SETUP :

| | | |
|---|---|---|
| Use pressure limitation function during loading and washing | ON | |
| Pressure limit for syringe pump | 160 digits | |
| Maximum count of triggered samples in series | 3 Sample /s | |
| System - rinsing and conditioning with solvent from port: | Port : | 1 MeOH |

Figure 2: Software Wizard (part 2) for the dual SPE method (automated)


Figure 3: Reusable plungers from LCTech GmbH allow higher operating pressure, higher flow-rates and reduced dead volume.

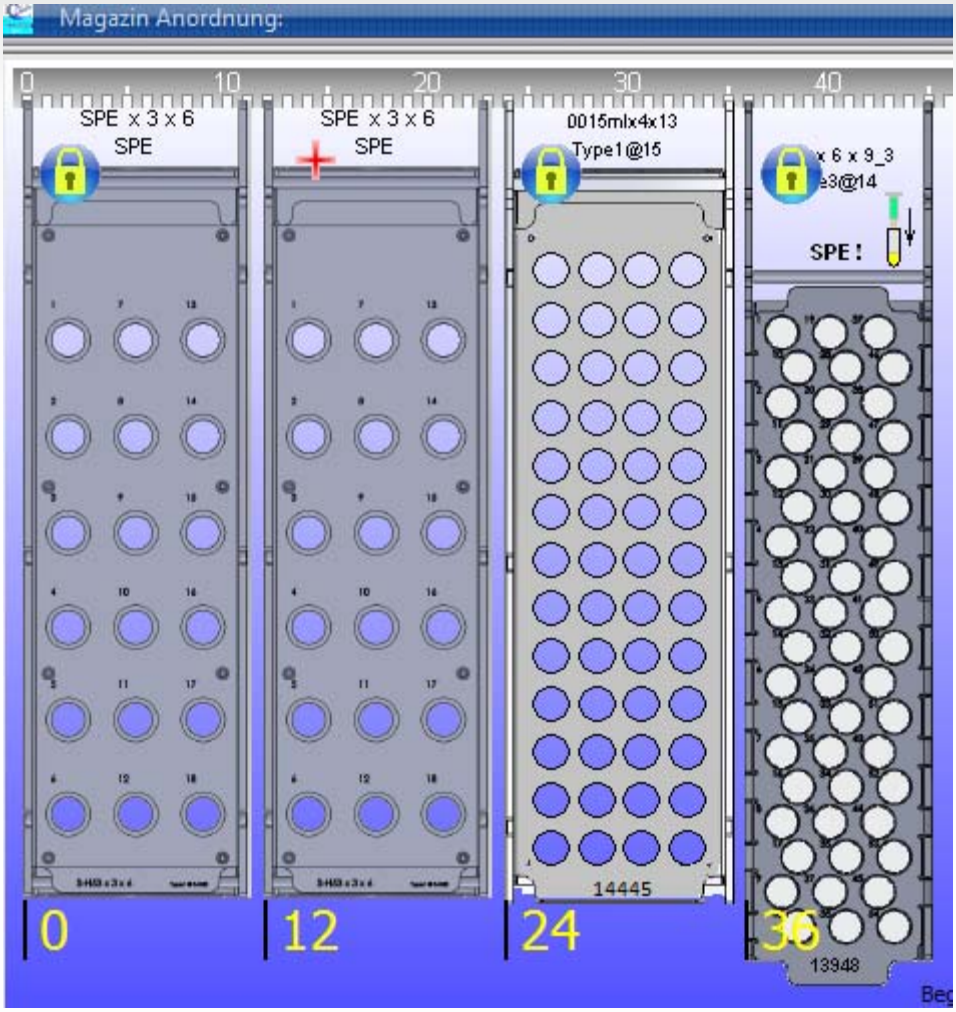


Figure 4: Rack arrangement for the dual SPE method (automated)

The eluate (from manual and automated clean-up) is dried in a gentle stream of nitrogen until dryness using a TurboVap from Biotage. After this step the sample is resolved in 500 µL of 0.1% formic acid in acetonitrile / 0.1 % formic acid in water (v/v, 10/90). The solution is measured using LC-MS/MS.

## 2.6 Measurement using HPLC-MS/MS

The HPLC-MS measurement can be performed on an Agilent 6495 combined with a 1290 Infinity Quat. Pump. The MRM-Transitions are shown in Table 4 and possible HPLC-MS/MS conditions are shown in Table 5. The sample extracts can also be measured using HPLC-HRMS (conditions not shown).

| Chemicals | $[M+H]^+$ | Q1 (Quantifier) | Q2 (Quantifier) |
|---|---|---|---|
| Alpha-Boldenone | 287 | 134.9 | 120.8 |
| Beta-Boldenone | 287 | 134.9 | 120.8 |
| Alpha-Nortestosterone | 275 | 109 | 90.8 |
| Beta-Nortestosterone | 275 | 109 | 90.8 |
| Alpha-Trenbolone | 271 | 199 | 164.9 |
| Beta-Trenbolone | 271 | 199 | 164.9 |
| Methyl-Testosterone | 303 | 108.9 | 96.9 |
| Stanozolol | 329 | 95 | 81 |
| Hydroxystanozolol | 345.1 | 96.8 | 66.8 |
| Ethinylestradiol | 279 | 158.9 | 132.8 |

Table 4: MRM-Transitions

| Column | Phenomenex Luna 3u C18(2) 100A, 150 x 2.00 mm |
|---|---|
| Eluent A | 0.1 % formic acid in acetonitrile |
| Eluent B | 0.1 % formic acid in water |
| Gradient | 10 % B to 93 % B (13 min.); 97 % B (hold 3 min.),<br>97 % B to 10 % B (0.5 min.), equilibration 3.5 min<br>Total run time 20 min. |
| Flow rate | 0.2 mL/min |
| Injection Volume | 10 µL |
| Column Temperature | 30 °C |
| Detection | Agilent 6495 with 1290 Infinity Quat. Pump<br>Ion source: ESI<br>Ionization mode: positive<br>Gas temperature: 200 °C<br>Gas flow: 11 L/min.<br>Nebulizer: 20 psi<br>Sheath gas heater: 380 °C<br>Sheath gas flow: 11<br>Capillary: 3000 V<br>VCharging: 1500<br>Ion funnel pos high pressure RF 150<br>Ion funnel pos low pressure RF 60 |

Table 5: Possible HPLC-MS/MS conditions

## 2.7 Configuration FREESTYLE

| | | |
|---|---|---|
| 1. | FREESTYLE BASIC | P/N 12663-12 |
| 2. | Liquid level switch for organic solvents | P/N 12709 |
| 3. | FREESTYLE SPE module | P/N 12668 |
| 4. | Rack for solvent delivery | P/N 13156 |
| 5. | Rack for SPE-columns (2 needed) for up to 18 columns | P/N 13946 |
| 6. | Tray (Elution for SPE) for 54 pcs / test tubes 100x16 mm | P/N 13948 |
| 7. | Sample rack for 52x15 mL tubes (Falcon/Saarstedt) | P/N 14445 |
| 8. | Plunger for 6 mL-cartridges (4 needed) (M&N, 10 pcs. / pck) | P/N 14596 |
| 9. | Plunger for 3 mL-cartridges (4 needed) (M&N, 10 pcs. / pck) | P/N 14597 |
| 10. | Column adapter for 6 mL SPE columns (4 needed) | P/N 14613 |
| 11. | Column adapter for 3 mL SPE columns (4 needed) | P/N 14612 |
| 12. | Plunger removal tool, 6 mL | P/N 14643 |
| 13. | Software upgrade “Extended functions” | P/N 14044 |
| 14. | FREESTYLE Software-Dongle | P/N 14111 |

# 3 Results

## 3.1 Recovery

Official laboratories in the European Union are obliged to detect 1 µg/kg of synthetic steroids in urine samples. This is easily achieved by using the manual as well as the automated application.

The recoveries achieved were better than the requirements (-50 % to + 20 % at concentrations < 1 µg/kg) set by the European law (decision 2002/657/EG) that means.

## 3.2 Reproducibility

Internal standards were added to ten different urine samples twice. The samples were purified manually using two SPE columns on the one hand. In the second approach the same samples were purified automatically using the robotic system FREESTYLE.

All 20 samples were measured using HPLC-HRMS (Thermo, Q-Exactive)

The absolute peak areas of the deuterated internal standards (concentration 3 µg/L urine) in the ten samples were compared.

Sample Results

| Sample Name | Excluded | Calculated Amt | Area | Expected RT | Actual RT |
|---|---|---|---|---|---|
| KAL 0 (0 ug/kg) | | 3.000 | 161260700 | 15.44 | 15.39 |
| KAL 1 (0.05 ug/kg) | | 3.000 | 158490589 | 15.44 | 15.39 |
| KAL 2 (0.1 ug/kg) | | 3.000 | 160560920 | 15.44 | 15.37 |
| KAL 3 (0.5 ug/kg) | | 3.000 | 159467625 | 15.44 | 15.37 |
| KAL 4 (1 ug/kg) | | 3.000 | 158529360 | 15.44 | 15.37 |
| KAL 5 (2 ug/kg) | | 3.000 | 155093991 | 15.44 | 15.36 |
| KAL 6 (4 ug/kg) | | 3.000 | 150667545 | 15.44 | 15.36 |
| KAL 7 (10 ug/kg) | | 3.000 | 138356012 | 15.44 | 15.34 |
| Blank | | 3.000 | 117887 | 15.44 | 15.34 |
| 2015MEL000703 b | | 3.000 | 42653979 | 15.44 | 15.36 |
| 2015MEL000704 b | | 3.000 | 43599181 | 15.44 | 15.34 |
| 2015MEL000705 b | | 3.000 | 53412056 | 15.44 | 15.34 |
| 2015MEL000706 b | | 3.000 | 36386635 | 15.44 | 15.33 |
| 2015MEL000710 b | | 3.000 | 39525642 | 15.44 | 15.32 |
| 2015MEL000877 b | | 3.000 | 61450110 | 15.44 | 15.31 |
| 2015MEL000878 b | | 3.000 | 58637548 | 15.44 | 15.60 |
| 2015MEL000879 b | | 3.000 | 54795571 | 15.44 | 15.31 |
| 2015MEL001057 b | | 3.000 | 29468347 | 15.44 | 15.30 |

Peak area mean: 46 658 785
Relative standard deviation: 23 %
Relative standard deviation based on the measurement system*: 5 %

Figure 5: Table with trenbolone reproducibility measurement results (automated via FREESTYLE)

Sample Results

| Sample Name | Excluded | Calculated Amt | Area | Expected RT | Actual RT |
|---|---|---|---|---|---|
| KAL 0 (0 ug/kg) | | 3.000 | 134932998 | 15.44 | 15.45 |
| KAL 1 (0.05 ug/kg) | | 3.000 | 132422820 | 15.44 | 15.44 |
| KAL 2 (0.1 ug/kg) | | 3.000 | 134256586 | 15.44 | 15.44 |
| KAL 3 (0.5 ug/kg) | | 3.000 | 134872252 | 15.44 | 15.43 |
| KAL 4 (1 ug/kg) | | 3.000 | 135563552 | 15.44 | 15.44 |
| KAL 5 (2 ug/kg) | | 3.000 | 134129367 | 15.44 | 15.45 |
| KAL 6 (4 ug/kg) | | 3.000 | 132651993 | 15.44 | 15.43 |
| KAL 7 (10 ug/kg) | | 3.000 | 122441471 | 15.44 | 15.44 |
| Blank | | 3.000 | 108184 | 15.44 | 15.45 |
| Blank | | N/F | N/F | 15.44 | N/F |
| 2015MEL000703 h | | 3.000 | 31680924 | 15.44 | 15.45 |
| 2015MEL000704 h | | 3.000 | 38051752 | 15.44 | 15.44 |
| 2015MEL000705 h | | 3.000 | 59861816 | 15.44 | 15.45 |
| 2015MEL000706 h | | 3.000 | 43476228 | 15.44 | 15.45 |
| 2015MEL000710 h | | 3.000 | 40881054 | 15.44 | 15.42 |
| 2015MEL000877 h | | 3.000 | 58796317 | 15.44 | 15.45 |
| 2015MEL000878 h | | 3.000 | 98294831 | 15.44 | 15.32 |
| 2015MEL000879 h | | 3.000 | 84798351 | 15.44 | 15.43 |
| 2015MEL001057 h | | 3.000 | 28588292 | 15.44 | 15.46 |

Peak area mean: 53 825 507
Relative standard deviation: 45 %
Relative standard deviation based on the measurement system*: 5 %

Figure 6: Table with trenbolone reproducibility measurement manually

*Relative standard deviation based on the measurement system was tested using the measurement of 8 standard solutions

| | FREESTYLE | | Manually | |
|---|---|---|---|---|
| | peak area mean | Relative standard deviation | peak area mean | Relative standard deviation |
| Boldenone | 17.718.489 | 21 | 18.656.051 | 32 |
| Nortestosterone | 87.055.332 | 18 | 95.172.536 | 29 |
| Stanozolol | 323.036.143 | 16 | 339.948.453 | 22 |
| Methyl testosterone | 161.171.697 | 19 | 184.326.218 | 21 |
| Ethinylestradiol | 488.678 | 18 | 647.134 | 39 |

Table 6: Reproducibility measurement results

## 4 Conclusions

A comparison of the results achieved manually and on the automated system, respectively, clearly shows that the sample clean-up for the analysis of synthetic steroids employing two SPE columns can easily be processed on the FREESTYLE SPE system.
The measured peak area mean values (Table 6) are very similar, and the relative standard deviations indicate a slightly better reproducibility for the automated processing.

## 5 Literature

Confirmatory Method for the Determination of Steroids in Bovine Urine by LC-MS/MS; Bundesamt für Verbraucherschutz und Lebensmittelsicherheit 2011

[1] S. Polemans, K. De Wasch, H. Noppe, N. Van Hoof, S. Van Cruchten, B. Le Bizec, Y. Deceunick, S. Sterk, H.J. Van Rossum, M.K. Hoffmann, H.F. Brabander; Endogenous occurence of some anabolic steroids in swine matrices; Food Additives and Contaminants, September 2005; 22(9):808-815

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