TITLE : URINE - THE ANALYSIS OF A SELECTION OF GROWTH PROMOTERS- LC-MS/MS

1 OBJECTIVE AND SCOPE

This SOP describes the analysis with LC-MS/MS of a selection of growth-promoting compounds: 17α–trenbolone, 17ß–trenbolone, androsta-1,4-diene-3,17-dione (ADD), 17ß–boldenone, 17α-boldenone, isoflupredone, dexamethasone, betamethasone, flumethasone, triamcinolone-acetonide, clobetasol, α-zeranol (α-zearalanol), β-zeranol (β-zearalanol), zearalanone, zearalenone, α-zearalenol, ß-zearalenol, and 16β-OH-stanozolol.

The method has a measuring range of 0 to 5.0 µg/l.

2 DEFINITION

MMS:	Matrix matched standards
ES	Electrospray
MS	Mass spectrometer
UPLC	Ultra Performance Liquid Chromatography
SPE	Solid Phase Extraction
ADD:	Androsta-1,4-diene-3,17-dione
16β-OH-stanozolol:	Metabolite of stanozolol
ACN:	Acetonitrile
MeOH	Methanol
HCOOH	Formic acid
Water	MilliQ H2O
DMSO	Dimethyl sulfoxide
HAc	Acetic acid
NH3	Ammonia
ISS	Internal standard solution
MSS	Mix standard solution

3 PRINCIPLE

Glucuronated compounds in urine are deconjugated by glucuronidase at pH 6.5 – 7.5 and 37 ^{0}C. After clean-up with a SPE the sample is measured by means of LC-MS/MS.

4 CHEMICALS AND REAGENTS

Any reference to type and/or product is only to inform the user and identify the equipment and does not imply exclusion of similar equipment.

All reagents and chemicals must be at least pro analysis quality.

With 'water' is meant water, purified with a MilliQ® system with a minimum resistance of at least 18.2 MΩ.cm-1.

Instead of listed below, chemical products from other suppliers may be used, as long as they are from comparable quality.

4.1 Reference Standards

4.1.1 17α–trenbolone (NMI D708)

4.1.2 17ß–trenbolone (Steraloids E3170-000)

4.1.3 17ß–Boldenone (Steraloids A200)

4.1.4 17α–Boldenone (Rikilt, EU/CRL 53)

This is an ampoule containing 0.1 mg 17α–boldenone

4.1.5 Isoflupredone (Research Plus 3005-16)

4.1.6 Flumethasone (Sigma F-9507)

4.1.7 Triamcinolone-acetonide (Sigma T-6501)

4.1.8 Betamethasone (Steraloids P0520-000)

4.1.9 Dexamethasone (Sigma D1756)

4.1.10 Clobetasol (Glaxo Wellcome)

4.1.11 ADD (androsta-1,4-diene-3,17-dione) (Steraloids A100)

4.1.12 α-Zeranol (Sigma Z-0292)

4.1.13 β-Zeranol (Sigma Z-0417)

4.1.14 Zearalanone (TRC Z270450)

This is a vial containing 1 mg zearalanone.

4.1.15 Zearalenone (Sigma Z-2125)

4.1.16 α-zearalenol (Sigma Z0166)

4.1.17 ß-zearalenol (Sigma Z 2000))

4.1.18 16β-OH-stanozolol NMI D621

This is an ampul containing 1 mg 16β-OH-stanozolol

4.1.19. Isoflupredone-d3 (d2 major) (TRC I816602)

This is a vial containing 1 mg Isoflupredone-d3 (d2 major).

4.1.20 17α-trenbolone-d3 (EURL 0101)

This is an ampoule containing 0.1 mg 17 α –boldenone-d3.

4.1.21 17ß–trenbolone-d3 (EURL 0102).

This is an ampoule containing 0.1 mg 17ß–trenbolone-d3.

4.1.22 17ß–Boldenone-d3 (EURL 0065)

This is an ampoule containing 0.1 mg 17ß–boldenone-d3.

4.1.23 α-zearalanol-d4/ ß-zearalanol-d4 (EURL 0006)

This is an ampoule containing 0.05 mg α-zearalanol-d4 and 0.05 mg / ß-zearalanol-d4

4.1.24 α-zearalenol-d4 (EURL 0048)

This is an ampoule containing 0,1 mg α-zearalenol-d4.

4.1.25 ß-zearalenol-d4 (EURL 0049)

This is an ampoule containing 0,1 mg ß-zearalenol-d4

4.1.26 16β-OH-stanozolol-d3 (EURL 0064)

This is an ampoule containing 0.1 mg 16β-OH-stanozolol-d3.

4.1.27 Triamcinolone-acetonide-d6 (EURL 0063)

This is an ampoule containing 0.1 mg triamcinolone-acetonide-d6.

4.1.28 Dexamethasone-d4 (Cachesyn, CSTD361)

4.1.29 Zearalenone-d6 (TRC Z270502)
This is an vial containing 1 mg zearalenone-d6.

4.1.30 Zearalanone-d6 (TRC 270442)
This is an vial containing 0.25 mg zearalanone-d6.

4.1.31 17ß–Nortestosterone-D3
Nortestosterone-D3 (EURL 0004) 19-nortestosterone (16,16,17-D3)
This is an ampoule containing 0.1 mg nortestosterone-D3

4.2 Chemicals
4.2.1 Natriumhydroxide (Merck 1.06498)

4.2.2 Acetonitrile (Biosolve 01203502)

4.2.3 Acetic acid (Merck 1.00063)

4.2.4 ß-Glucuronidase E Coli K12 (Roche 03707598001)

4.2.5 Methanol (Biosolv 13683502)

4.2.6 Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)(Merck 1.06580)

4.2.7 Potassium dihydrogen phosphate (KH_2PO_4)(Merck 1.04873)

4.2.8 Ammonium formate (Sigma 156264)

4.2.9 Formic acid (Merck 264)

4.2.10 Ethanol (Merck 1.00983)
4.2.11 Ammonia 25% (Merck 1.05432)

4.3 Standard solutions
Prepare standard solutions of individual compounds and internal standards. The concentrations, solvents, shelve lives (in freezer) and the amounts to weigh are given in table 1.

Table 1: Solvent and concentrations of stock solutions

Compound	Amount standard (mg)	Concentration stock solution (mg/l)	Solvent stock solution	Shelve lives (months)
17α–Trenbolone (4.1.1)	2 - 10	1000	methanol	12
17ß–Trenbolone (4.1.2)	2 - 10	1000	methanol	12
17ß–Boldenone (4.1.3)	2 - 10	1000	methanol	12
17α–Boldenone (4.1.4)	Ampul*	10	methanol	12
Isoflupredone (4.1.5)	2 - 10	1000	methanol	12
Flumethasone (4.1.6)	2 - 10	1000	methanol	12
Triamcinolone-acetonide (4.1.7)	2 - 10	1000	methanol	12
Betamethasone (4.1.8)	2 - 10	1000	methanol	12
Dexamethasone (4.1.9)	2 - 10	1000	methanol	24
Clobetasol (4.1.10)	2 - 10	1000	methanol	12
ADD (4.1.11)	2 - 10	1000	methanol	12
α-Zearalanol (4.1.12)	2 - 10	1000	methanol	24
β-Zearalanol (4.1.13)	2 - 10	1000	methanol	24
Zearalanone (4.1.14)	1	100	methanol	24
Zearalenone (4.1.15)	2 - 10	1000	methanol	24
α-Zearalenol (4.1. 16)	2 - 10	1000	methanol	24
ß-Zearalenol (4.1.17)	2 - 10	1000	methanol	24
16ß-OH-stanozolol (4.1.18)	Ampul*	100	methanol	24
Isoflupredone-d3 (4.1.19)	1	100	methanol	24
17α-Trenbolone-d3 (4.1.20)	Ampul*	10	methanol	24
17ß–Trenbolone-d3 (4.1.21)	Ampul*	10	methanol	24
17ß–Boldenone-d3 (4.2.22)	Ampul*	10	ethanol	24
α-Zearalanol-d4/β-zearalanol-d4 (4.1.23)	Ampul**	10	methanol	24
α-Zearalenol-d4 (4.1.24)	Ampul*	10	ethanol	24
ß-Zearalenol-d4 (4.1.25)	Ampul*	10	ethanol	24
16β-OH-stanozolol-d3 (4.1.26)	Ampul*	10	methanol	24
Triamcinolone-acetonide-d6 (4.1.27)	Ampul*	10	methanol	24
Dexamethasone-d4 (4.1.28)	2-10	1000	methanol	24
Zearalenone–d6 (4.1.29)	1	100	methanol	24
Zearalanone-d6 (4.1.30)	0.25	25	methanol	24
17ß–Nortestosterone-D3 (4.1.31)	Ampul*	10	methanol	24

* Open the ampoule and add 1 ml solvent. Place during 1 minute in a ultra sonic bath, mix and transfer the contents by means of the solvent quantitative in a 10 ml flask. Fill up with solvent and mix.
** Open the ampoule and add 1 ml solvent. Place during 1 minute in a ultra sonic bath, mix and transfer the contents by means of the solvent quantitative in a 5 ml flask. Fill up with solvent and mix.

4.3.2 MSS I 10 mg/l

Pipet the amount of 100 µl of each stock solution of 1000 mg/l and 1000 µl of each stock of 100 mg/l in volumetric flasks of 10 ml and make up to volume with MeOH. The standard solutions are stored in a freezer for a maximum period of 12 months.

4.3.3 MSS II 0.1 mg/l

Pipet 100 µl of MSS I (4.3.2), 100 µl of each stock solution 10 mg/l and 10 µl of each stock solution 100 mg/l in a volumetric flasks of 10 ml and make up to volume with MeOH. The standard solution is stored in a freezer for a maximum period of 3 months.

4.3.4 MSS III 0.02 mg/l

Pipet 2 ml of MSS II (4.3.3) in a volumetric flasks of 10 ml and make up to volume with MeOH. The standard solution is stored in a freezer for a maximum period of 3 months.

4.3.5 MSS IV 0.002 mg/l

Pipet 200 µl of MSS II (4.3.3) in a volumetric flasks of 10 ml and make up to volume with MeOH. The standard solution is stored in a freezer for a maximum period of 3 months.

4.3.6 ISS dexamethasone-d4 10 mg/l

Pipet 100 µl of stock solution 1000 mg/l in a volumetric flasks of 10 ml and make up to volume with MeOH. The standard solution is stored in a freezer for a maximum period of 12 months.

4.3.7 ISS I 0.1 mg/l

Pipet 100 µl of ISS dexamethasone-d4 10 mg/l (4.3.6), 100 µl of all other internal standards 10 mg/l, 40 µl of internal standard 25 mg/l and 10 µl of internal standard 100 mg/l in a volumetric flasks of 10 ml and make up to volume with MeOH. The standard solution is stored in a freezer for a maximum period of 12 months.

4.3.8 Working solution standards

Pipet 60 µl of MSS II 0.1 mg/l (4.3.3) and 60 µl of ISS I 0.1 mg/l (4.3.7) in a glass tube. Evaporate at 40 °C ± 10% under a gentle stream of nitrogen until dry and re-dissolve in 120 µl DMSO and 180 µl 10% MeOH.

4.4 Reagents

4.4.1 HAc 8 N

Dilute 46 ml HAc to 100 ml with water. This solution is stable for 3 months at room temperature.

4.4.2 NaOH 8 N

Dissolve 32.0 gram NaOH in water and add water to a final volume of 100 ml. This solution is stable for 3 months at room temperature

4.4.3 Na_2HPO_4 0.2 M

Dissolve 3.56 g $Na_2HPO_4.2H_2O$ in water and add water to a final volume of 100 ml. This solution is stable for 1 month at room temperature.

4.4.4 KH_2PO_4 0.2 M

Dissolve 2.72 g 4 KH_2PO_4 in water and add water to a final volume of 100 ml. This solution is stable for 1 month at room temperature.

4.4.5 0.2 M Phosphate buffer pH 7.0

Add KH_2PO_4 0.2 M under stirring to Na_2HPO_4 0.2 M till pH is 7.0 ± 0.2 (pH meter). This solution is stable for 1 month at room temperature.

4.4.6 Ammonium formate 1 M

Dissolve 6.3 g ammonium formate in water and add water to a final volume of 100 ml. This solution is stable for 3 months at room temperature

4.4.7 Wash solvent 1: 60% MeOH/2% HAc

Mix 38 ml water, 60 ml MeOH and 2 ml HAc. This solution is stable for 3 months at room temperature.

4.4.8 Wash solvent 2: 20% ACN/2%HAc

Mix 78 ml water, 20 ml ACN and 2 ml HAc. This solution is stable for 3 months at room temperature.

4.4.9 Wash solvent 3: 20% ACN

Mix 80 ml water with 20 ml ACN. This solution is stable for 3 months at room temperature.

4.4.10 Wash solvent 4: 10% ACN/2%NH3

Mix 82 ml water, 8 ml ammonia (25%) and 10 ml ACN. This solution is stable for 3 months at room temperature.

4.4.11 Wash solvent 5: 50%MeOH/2% NH3

Mix 42 ml water, 8 ml ammonia (25%) and 50 ml MeOH. This solution is stable for 3 months at room temperature.

4.4.12 Wash solvent 6: 60% MeOH

Mix 40 ml water and 60 ml MeOH. This solution is stable for 3 months at room temperature.

4.4.13 Mobile phase A: water/ACN/HCOOH/ammonium formate 1 M 900/100/0.02/2 (v/v/v/v)
Mix 900 ml water, 100 ml ACN, 0.02 ml HCOOH and 2 ml ammonium formate 1 M (4.4.6). This solution is stable for 1 month at room temperature.

4.4.14 Mobile phase B: water/ACN/HCOOH/ammonium formate 1 M 100/900/0.02/2 (v/v/v/v)
Mix 100 ml water, 900 ml ACN, 0.02 ml HCOOH and 2 ml ammonium formate 1 M (4.4.6). This solution is stable for 1 month at room temperature.

5 EQUIPMENT
Any reference to type and/or product is only to inform the user and identify the equipment and does not imply exclusion of similar equipment.

5.1 Analytical balance with an accuracy of 0.02 mg or better (Mettler AT 261)

5.2 pH indicator strips 6.5 – 10,0 (Merck 9543)

5.3 Water bath adjustable at 37 ^{0}C (Julabo MD/25B)

5.4 Glass tube 12 ml (Beldico, 8739007)

5.5 Polypropylene tube 12 ml with screwcap (Greiner 163275)

5.6 Evaporation unit (Turbo-Vap LV Zymark 44467)

5.7 Evaporator for 96 wells (Caliper LifeSciences Turbovap 96)

5.8 Vortex (IKA Vibrofix VF1)

5.9 Vortex (IKA Genius 3)

5.10 OASIS HLB LP 96-well plate 60 µm (60mg) (Waters 186000679)

5.11 Extraction plate manifold for Oasis 96-wells plates (Waters 186001831)

5.12 Vacuum pump (KNF Lab Laboport D79112)

5.13 96-Well collection plates for Waters Acquity UPLC I-Class, well volume 2 ml (WAT 058985)

5.14 96-Well PTFE/Silicone seal with pre-slit (Waters 186006335)

5.15 12-Channel pipette (Rainin 100-1200 µl LTS Pipet Lite, L12-1200XLS, Mettler Toledo 17014497)

5.16 pH meter (Scott CG 840)

5.17 LC–MS opstelling

5.17.1 Waters Acquity pump and injection system

5.17.2 Analytical kolom: Acquity UPLC BEH C_{18}, 100 mm x 1.0 mm, 1.7 µm (Waters 186002346)

5.17.3 Mass spectrometer: Waters, Xevo TQS with ESI interface.

6 PROCEDURE

6.1 General

This SOP describes the quantification and confirmation of a selection of growth promoters in cattle urine.

6.2 Safety precautions

Take precautions to prevent inhalation and contact with skin from standards and chemicals. Work in a fume hood and wear gloves if necessary (SOP F0071). [2].

6.3 Sample pre-treatment

Frozen samples are thawed at room temperature or overnight in the fridge. Homogenize samples before a test unit is taken. After that place sample as soon as possible back in the freezer.

6.4 Test unit

Take 0.5 ml of urine.

6.5 Description procedure

A checklist of the method is given in appendix 2.

6.5.1 Composition of samples series

A serie consists of samples, 7 samples with spike before processing (MMS) and a 1st–line control. For the MMS, and 1st–line control a urine sample is used which in previous analysis no compounds were detected. For the 1st–line control another blank urine is taken than used for MMS.

6.5.2 Preparing MMS,1st–line control and urine samples

6.5.2.1 Preparing MMS

Pipet in separate polypropylene tubes the MSS and ISS according to table 2.

Table 2. Preparing MMS

Matrix Matched Standards	µl MSS IV (0.002 mg/l)(4.3.5)	µl MSS III (0.02 mg/l)(4.3.4)	µl MSS II (0.1 mg/l)(4.3.3)	µl ISS I 0.1 mg/l (4.3.7)
MMS A (0 µg/l)	0			25
MMS B (0.1 µg/l)	25			25
MMS C (0.2 µg/l)	50			25
MMS D (0.5 µg/l)		12.5		25
MMS E (1.0 µg/l)		25		25
MMS F (2.0 µg/l)		50		25
MMS G (5.0 µg/l)			25	25

Evaporate under a gently stream of nitrogen at 40 °C ± 10% until just dry. Pipet in every tube a test unit of a blank sample. Mix with vortex. Proceed from 6.5.3.

6.5.2.2 1st–line control

Pipet in polypropylene tube 25 µl of ISS I (0.1 mg/l)(4.3.7) and 25 µl MSS III (0.02 mg/l)(4.3.4). Evaporate under a gently stream of nitrogen at 40 °C ± 10% until just dry. Pipet 1 test unit of another blank urine than used for MMS in the polypropylene tube. Mix with vortex. Proceed from 6.5.3.

6.5.2.3 Preparing samples

Pipet for each sample in separately polypropylene tubes 25 µl of ISS I (0.1 mg/l)(4.3.7). Evaporate under a gently stream of nitrogen at 40 °C ± 10% until just dry. Pipet for each samples a test unit in the polypropylene tube. Mix with vortex. Proceed from 6.5.3.

6.5.3 Deconjugation

Add to each tube 500 µl 0.2 M phosphate buffer pH 7.0 (4.4.5) and vortex. Check the pH with pH indicator strips and adjust if necessary with 8M NaOH and/or 8M HAC or diluted solutions thereof until the pH 6.5-7.5 is. Add 5 µl ß-glucuronidase and mix. Place in a water bath at 37 ^{0}C during 16 hours. Cool to room temperature.

6.5.4 Sample clean-up by 96-wells SPE

Conditioning a SPE plate with 1 ml of MeOH followed by 1 ml of water. Bring whole sample in SPE well. Wash with 1 ml water and dry. Wash successively with 1 ml wash solvent 1 to 6. Dry after each wash step. Elute with 1 ml ACN. Collect eluate in a 96-well collection plate which in each well is already 20 µl of DMSO. Place plate in a 96-wells evaporator and dry at 60 °C ± 10% till only DMSO is left. Add 30 µl of 10% MeOH and vortex (5.9) carefully. The samples are now suitable for LC-MS/MS analysis.

6.5.5 LC-MSMS analysis

6.5.5.1 LC-conditions

Column :	UPLC BEH C_{18}, 100 mm x 1.0 mm, 1.7 µm
Mobile phase A:	water/ACN/HCOOH/ammonium formate 1 M 900/100/0.02/2 (v/v/v/v)
Mobile phase B:	water/ACN/HCOOH/ammonium formate 1 M 100/900/0.02/2 (v/v/v/v)
Column temperature:	60 °C
Vialtray temperature:	20°C
Injection volume	5 µl
Flow:	0.15 ml/min
Sample manager purge:	H_2O/ACN 700/300 (v/v)
Sample manager wash:	ACN/water/HCOOH 900/100/0.1 (v/v/v)
Seal wash:	H_2O/MeOH 900/100 (v/v)
Gradiënt:	see table 3

Table 3: Gradient

Time (min)	Mobiele fase A (%)	Mobiele fase B (%)	Flow (ml/min)
0.0	80	20	0.15
0.2	80	20	0.15
3.2	70	30	0.15
7.5	50	50	0.15
7.6	0	100	0.15
8.6	0	100	0.15
8.7	80	20	0.15
10.0	80	20	0.15

6.5.5.2 MS-condities

The MS parameters are guidelines. To obtain a better performance these parameters can be changed.

Ionisation mode: ESI, negative and positive

Parameter	ESI, positive	ESI, negative*
Capillair voltage	3.0	2.2
LM 1 resolution	3.0	2.8
HM 1 resolution	15.0	15.0
Ion energy 1	1.0	1.9
LM 2 resolution	3.0	3.0
HM 2 resolution	15.0	15.0
Ion energy 2	1.0	1.3

* = specific values for TQS1

Cone:	compound depends V
Source temperature:	150 °C
Desolvation temperature:	400 °C
Desolvation gas flow:	800 l/hour
Cone gas flow:	150 l/hour
Nebuliser gas flow	7 bar
CID gas:	argon, p = 2,2 * 10^{-3} mbar, purity > 99.998%
CID gas flow:	0.18 ml/min

The compounds fragment to structure related product ions. In table 4 the theoretical mono-isotopic masses of the precursor ions and corresponding product ions are displayed. For each of the mono-isotopic masses the permitted deviation is ± 0.5 Da.

Table 4: Monitored MRM's

Component	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collission energy (eV)	ESI
Isoflupredone*	423.2	293.0	20	30	-
		347.1		15	
Isoflupredone-d3*	426.2	350.1	20	17	-
Dexamethasone/Betamethasone*	437.2	307.0	20	30	-
		361.2		15	
Dexamethasone-d4*	441.2	363.10	20	15	-
Flumethasone*	455.2	305.0	20	30	-
		379.1		20	
17ß–Trenbolone/17α–Trenbolone	271.2	199.1	30	*25*	+
		253.2		*20*	
17ß–Trenbolone-d3/17α–Trenbolone-d3	274.1	256.2	30	20	+
17ß–Boldenone/17α–Boldenone	287.2	121.1	25	25	+
		135.1	30	15	
17ß–Boldenone-d3	290.2	121.1	25	25	+
ADD	285.2	121.1	25	25	+
		147.1	15	15	

Component	Precursor ion (m/z)	Product ion (m/z)	Cone Voltage (V)	Collission energy (eV)	ESI
16β-OH-Stanozolol	345.2	81.1	30	45	+
		95.1		40	
16β-OH-Stanozolol-d3	348.3	81.1	50	45	+
Triamcinolone-acetonide	435.3	339.2	40	13	+
		415.3		8	
Triamcinolone-acetonide-d6	441.3	421.3	40	8	+
Clobetasol	411.2	355.1	20	13	+
		391.2		5	
Zearalenone	317.2	131.2	44	30	-
		175.1		24	
ß-Zearalenol/α-Zearalenol/Zearalanone	319.2	160.2	30	30	-
		275.2		21	
ß-Zearalenol-d4/α-zearalenol-d4	323.2	160.1	45	32	-
Zearalanone	319.2	205.2	54	22	
ß-Zearalanol/α-Zearalanol	321.2	259.2	56	26	-
		277.2		20	
β-Zearalanol-d4/α-Zearalanol-d4/Zearalanone-d6	325.1	281.1	50	20	-
17ß-Nortestosterone-d3	278.2	109.1	30	25	+

* = formic acid adduct

6.5.5.3 Initial test LC system

By means of working standard solution (4.3.8) the retention time and stability of the system are tested. Inject hereafter MMS B. Calculate the signal/noise ratio of the lowest ion of each compound. The signal/noise ratio should be at least 6 for each compound.

6.5.5.4 Injection order

The following injection order is advisable:

-MMS B (initial test)

-blank

-MMS A - G

-blank

-1st-line control

-blank

-samples

-blank

- MMS A – G

7 RESULTS

7.1 Calculations

Table 5 is an overview of which internal standard is used for calculations.

Table 5: Compound with internal standard used for calculations

Compound	Internal standard
ADD	17ß–Nortestosterone-D3
Isoflupredone	Isoflupredone-d3
Dexamethasone/Betamethasone	Dexamethasone-d4
Flumethasone	Dexamethasone-d4
17ß–Trenbolone	17ß–Trenbolone-d3
17α–Trenbolone	17α–Trenbolone-d3
17ß–Boldenone	17ß–Boldenone-d3
17α–Boldenone	17ß–Boldenone-d3
Triamcinolone-acetonide	Triamcinolone-acetonide-d6
16β-OH-Stanozolol	16β-OH-stanozolol-d3
Clobetasol	Dexamethasone-d4
Zearalenone	α-Zearalenone-d6
ß-Zearalenol	ß-Zearalenol-d4
α-Zearalenol	α-Zearalenol-d4
Zearalanone	α-Zearalanone-d6
ß-Zearalanol	β-Zearalanol-d4
α-Zearalanol	α-Zearalanol-d4

Equation I: Calculation response factor (RF)

$$RF = \left(\frac{Area_{compound}}{Area_{IS}} \right)$$

With:

RF = response factor

Area$_{compound}$ = sum area product ions

Area$_{IS}$ = area product ion internal standard

Equation II: Calculation of the concentration in the sample (X)

$$X = \left(\frac{RF - b}{a} \right)$$

With:

X = concentration of a compound in the sample (µg/l)

RF = response factor (equation I (7.1)).

b = intersection point of the calibration curve with the y-axis (follows from linear regression*)

a = slope of the calibration line (follows from linear regression*)

*Plot the area of the MMS measured before and after the sample extracts as function of the added levels. Calculate the linear regression using the least squares method.

Equation III: Calculation relative retention time (RRT)

$$RRT = \left(\frac{RT_{compound}}{RT_{IS}} \right)$$

With:

RRT = relative retention time

$RT_{compound}$ = retention time of compound

RT_{IS} = retention time of internal standard

Equation IV: Calculation of the relative deviation of the relative retention time (ΔRRT)

$$\Delta RRT = \left(\frac{RRT_{sample} - RRT_{mean}}{RRT_{mean}} \right) \times 100\%$$

With:

ΔRRT = relative deviation of the relative retention time of compound compare to mean retention time of MMS B up to and including G before and after samples (%)

RRT_{sample} = relative retention time of a compound in the sample

$RRT_{mean.}$ = mean relative retention time of a compound in the MMS B up to and including G before and after samples

Equation V: Calculation minimal respons of internal standard for assurance determination

$$A_{\min} = \frac{6 * A_{MMS\_RL}}{S / N}$$

With:

A_{min} = minimal of internal standard in a sample where level of ¼ of the proposed target is guaranteed

A_{MMS_RL} = lowest area of internal standard of MMS B before or after samples

S/N = signal/noise ratio of the lowest product ion of MMS B before the samples

Equation VI: Ion ratio (R):

$$R = \left(\frac{A_{low}}{A_{high}} \right) \times 100\%$$

With:

R = ion ratio (%)

A_{low} = area of the product ion with the lowest intensity

A_{high} = area of the product ion with the highest intensity

Equation VII: Relative deviation of the ion ratio (D):

$$\Delta R = \left(\frac{\left(R_{sample} - R_{mean.} \right)}{R_{mean}} \right) \times 100\%$$

With:

D = relative deviation of the ion ratio of the compound in the sample compared to the average ion ratio of the compound in MMS B up to and including G before and after samples

R_{sample} = ion ratio of the compound in the sample

Rmean = mean ion ratio of the compound in MMS B up to and including G before and after samples

7.2 Criteria

7.2.1 General

For acceptance of the analysis results, the performance criteria (stated below) have to be met. The concentrations are automatically calculated when the result sheet (appendix 1) is used. At the same time the excel sheet will show if the calculated analysis results meet the stated criteria.

7.2.2 Drift in sensitivity

The MMS analysed before the sample extracts compared with the MMS analysed after the sample extracts are indicative for the performance of the LC-MS/MS system. For both MMS series, the sum of the area of the product ions is plotted as function of the added concentration to the sample (µg/l). Two slopes for both MMS series are constructed, which may not differ by more than 25%.

7.2.3 Linearity

The MMS are used to determine the linearity of the LC-MS/MS system and to determine if the sample pre-treatment is done correctly. For both MMS series, the sum of the area of the product ions is plotted as function of the added concentration in the sample (µg/l). Apply linear regression using the least squares method. The correlation of both lines should be ≥ 0.990.

7.2.4 Sensitivity

The S/N of the least intense product ion of MMS 0.25*RW should be ≥ 6.

7.2.5 Accuracy

Calculate the concentration in the 1st line control using equation II. Calculate the accuracy for each compound by dividing the concentration by the actual spiked level (%). Fill in the Shewart charts. SOP F0057 discusses Shewhart charts.

7.2.6 Maximum deviation retention time

Determine the average retention time of the compounds in MMS B - G before and after samples the sample extracts. The deviation in the individual retention times may not differ more than 2.5% compared to the mean retention time of the compounds in the MMS B - G.

7.2.7 Maximum deviation ion ratio

Calculate the mean ion ratio of the MMS B up to and including G before and after the samples. The deviation in the ion ratio of each MMS sample compared to the mean should not exceed the EU-criteria in table 6.

Table 6: Maximum permitted tolerances for relative ion intensities according to EU criteria [3]

Average ion ratio MMS (R)	Permitted relative deviation (D)
R > 50%	≤20%
20% < R ≤ 50 %	≤25%
10% < R ≤ 20 %	≤30%
R ≤ 10%	≤50%

7.2.8 Quantification

The concentration of the compounds in the sample is expressed as µg/l and is calculated using the linear regression curves calculated from the MMS series analysed before and after the sample extracts. Calculate the concentrations using equation I and II (7.1). The concentration is automatically calculated using the result sheet (appendix 1).

7.2.9 Criteria for individual samples

Calculate for each sample the relative deviation of the retention time and the relative deviation of the ion ratio.

When the relative deviation of the retention time does not exceed 2.5% and the relative deviation of the ion ratio does not exceed the EU-criteria and the concentration exceeds CCα, the sample is non-compliant. The second and/or third validation level decides whether the sample is reanalyzed.

7.2.10 Accuracy

Calculate the concentration of a compound in the 1st–line control sample using equation II. Calculate the accuracy for each compound by dividing the concentration by the actual spiked level (%). Fill in the Shewart charts. SOP F0057 discusses Shewart charts

7.3 Final results

When a compound is identified in the sample with a concentration exceeding CCα, the concentration is reported in LIMS. When no compound is identified in the sample "<0.25 RW" (lowest MMS) is reported in LIMS.

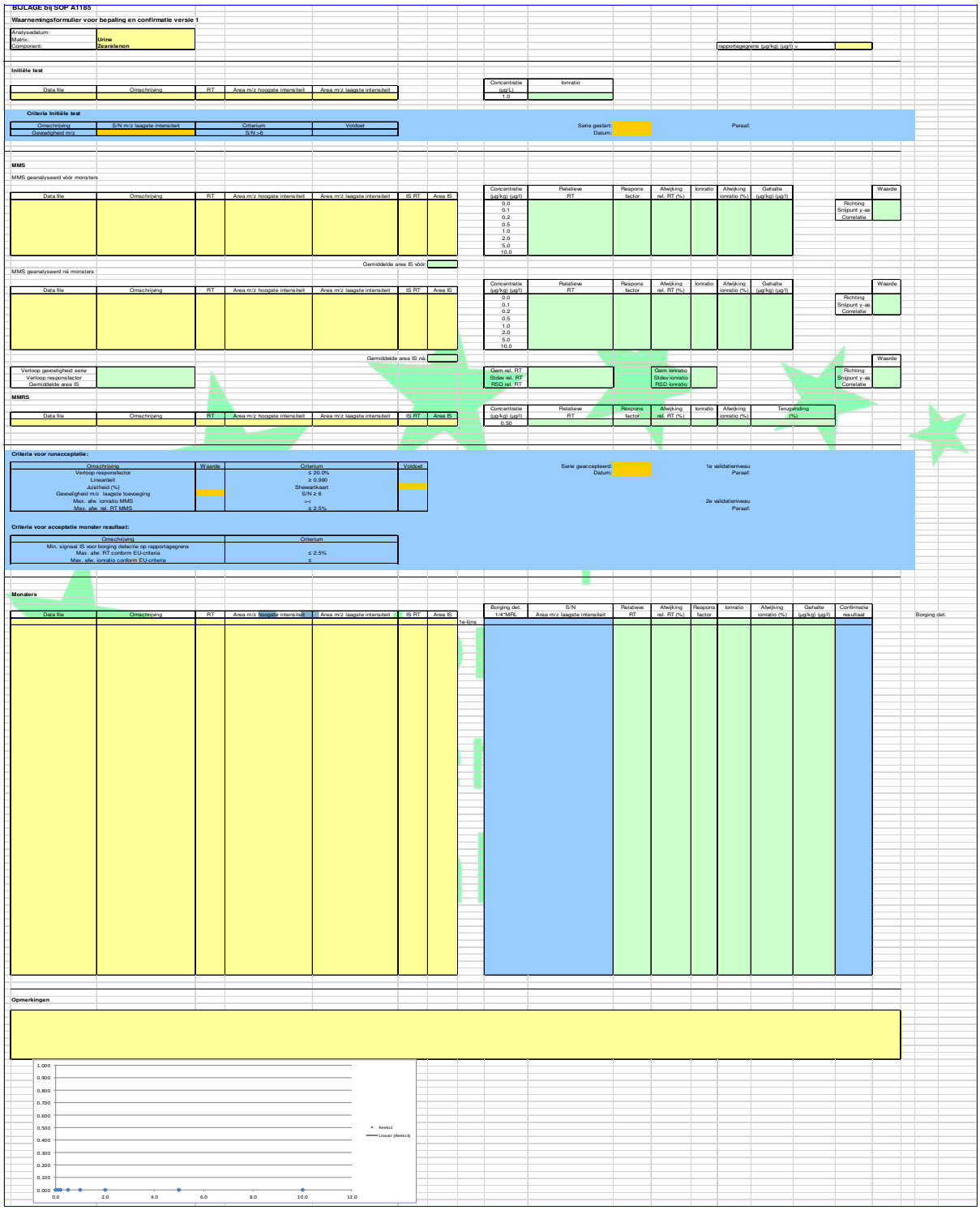
8 REGISTRATION

All information concerning the analysis is described in the labjournal. The MS/MS data are digital archived.

LITERATURE

[1] EU Beslissing 2002/657/EC, J.EUR. Communities, L221 (2002) 8
[2] SOP F0057 – Controle kaarten – Gebruik en beheer
[3] SOP F0071 – Persoonlijke beschermingsmiddelen binnen het RIKILT

Appendix 1 belonging to SOP A1185



BIJLAGE bij SOP A1185

Waarnemingsformulier voor bepaling en confirmatie versie 1

Analysedatum:
Matrix: **Urine**
Component: **Zearalanon**

rapportagegrens (µg/kg) (µg/l) =

Initiële test

Data file	Omschrijving	RT	Area m/z hoogste intensiteit	Area m/z laagste intensiteit

Concentratie (µg/L)	Ionratio
1.0	

Criteria Initiële test

Omschrijving	S/N m/z laagste intensiteit	Criterium	Voldoet
Gevoeligheid m/z		S/N >6	

Serie gestart:
Datum:
Parsaat:

MMS

MMS geanalyseerd vóór monsters

Data file	Omschrijving	RT	Area m/z hoogste intensiteit	Area m/z laagste intensiteit	IS RT	Area IS

Gemiddelde area IS vóór

Concentratie (µg/kg) (µg/l)	Relatieve RT	Respons factor	Afwijking rel. RT (%)	Ionratio	Afwijking ionratio (%)	Gehalte (µg/kg) (µg/l)
0.0						
0.1						
0.2						
0.5						
1.0						
2.0						
5.0						
10.0						

	Waarde
Richting	
Snijpunt y-as	
Correlatie	

MMS geanalyseerd ná monsters

Data file	Omschrijving	RT	Area m/z hoogste intensiteit	Area m/z laagste intensiteit	IS RT	Area IS

Gemiddelde area IS ná

Concentratie (µg/kg) (µg/l)	Relatieve RT	Respons factor	Afwijking rel. RT (%)	Ionratio	Afwijking ionratio (%)	Gehalte (µg/kg) (µg/l)
0.0						
0.1						
0.2						
0.5						
1.0						
2.0						
5.0						
10.0						

	Waarde
Richting	
Snijpunt y-as	
Correlatie	

Verloop gevoeligheid serie	
Verloop responsfactor	
Gemiddelde area IS	

Gem rel. RT	
Stdev rel. RT	
RSD rel. RT	

Gem ionratio	
Stdev ionratio	
RSD ionratio	

	Waarde
Richting	
Snijpunt y-as	
Correlatie	

MMRS

Data file	Omschrijving	RT	Area m/z hoogste intensiteit	Area m/z laagste intensiteit	IS RT	Area IS

Concentratie (µg/kg) (µg/l)	Relatieve RT	Respons factor	Afwijking rel. RT (%)	Ionratio	Afwijking ionratio (%)	Terugvinding (%)
0.50						

Criteria voor runacceptatie:

Omschrijving	Waarde	Criterium	Voldoet
Verloop responsfactor		≤ 20.0%	
Lineariteit		≥ 0.990	
Juistheid (%)		Shewartkaart	
Gevoeligheid m/z laagste toevoeging		S/N ≥ 6	
Max. afw. ionratio MMS		=<	
Max. afw. rel. RT MMS		≤ 2.5%	

Serie geaccepteerd:
Datum:

1e validatieniveau
Parsaat:

2e validatieniveau
Parsaat:

Criteria voor acceptatie monster resultaat:

Omschrijving	Criterium
Min. signaal IS voor borging detectie op rapportagegrens	
Max. afw. RT conform EU-criteria	≤ 2.5%
Max. afw. ionratio conform EU-criteria	≤

Monsters

Data file	Omschrijving	RT	Area m/z hoogste intensiteit	Area m/z laagste intensiteit	IS RT	Area IS

1e-lijns

Borging det. 1/4*MRL	S/N Area m/z laagste intensiteit	Relatieve RT	Afwijking rel. RT (%)	Respons factor	Ionratio	Afwijking ionratio (%)	Gehalte (µg/kg) (µg/l)	Confirmatie resultaat

Borging det.

Opmerkingen

Appendix 2 belonging to SOP A1185 **Checklist**

Date: _____
Name analist: _____
Labjournal/page: _____
Working list number(s) _____

☐ Preparing MMS. 1st-line control and samples

Matrix Matched Standards	µl MSS IV (0.002 mg/l)(4.3.5)	µl MSS III (0.02 mg/l)(4.3.4)	µl MSS II 0.1 mg/l)(4.3.3)	µl ISS 0.1 mg/l (4.3.7)
MMS A (0 µg/l)	0			25
MMS B (0.1 µg/l)	25			25
MMS C (0.2 µg/l)	50			25
MMS D (0.5 µg/l)		12.5		25
MMS E (1.0 µg/l)		25		25
MMS F (2.0 µg/l)		50		25
MMS G (5.0 µg/l)			25	25
1st-line control		25		25
Samples				25

☐ Evaporate until just dry at 40 ^{0}C
☐ Pipet 0.5 ml blank urine (1st line control another urine than MMS)
☐ Pipet 0.5 ml of each sample in tube
☐ Add 500 µl 0.2 M phosphatebuffer pH 7.0
☐ Mix and adjust to pH 6.5 - 7.5 with HAC/NaOH
☐ Add 5 µl ß-Glucuronidase and mix
☐ Place during 16 hours in water bath at 37 ^{0}C
☐ Cool to room temperature
☐ Conditioning a SPE plate with 1 ml of MeOH followed by 1 ml of water
☐ Transfer whole sample in SPE well
☐ Wash with 1 ml of water and dry
☐ Wash with 1 ml wash solvent 1 and dry
☐ Wash with 1 ml wash solvent 2 and dry
☐ Wash with 1 ml wash solvent 3 and dry
☐ Wash with 1 ml wash solvent 4 and dry
☐ Wash with 1 ml wash solvent 5 and dry
☐ Wash with 1 ml wash solvent 6 and dry
☐ Pipet 20 µl DMSO in each well of the collection plate
☐ Elute with 1 ml ACN.
☐ Place plate in a 96-wells evaporator and dry at 60 °C ± 10% till only DMSO is left.

- □ Add 30 µl of 10% MeOH.and vortex (5.9) carefully
- □ The samples are now suitable for LC-MS/MS analysis