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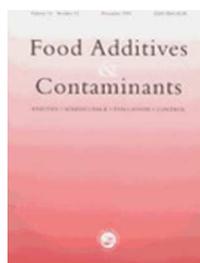
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Endogenous steroid profiling by gas-chromatography tandem mass spectrometry and multivariate statistics for detection of natural hormone abuse in cattle

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Methods/Techniques:	Chromatography - GC/MS, GC/MS, Statistical analysis
Additives/Contaminants:	Drug residues - hormones
Food Types:	Animal products – meat
Abstract:	<p>For years it is suspected that natural hormones are illegally used as growth promoters in cattle in the European Union. Unfortunately there is a lack of methods and criteria that can be used to detect the abuse of natural hormones and distinguish treated from non-treated animals. Pattern recognition of steroid profiles is a promising approach for tracing/detecting the abuse of natural hormones administered to cattle. Traditionally steroids are analyzed in urine as free steroid after deconjugation of the glucuronide (and sulphate) conjugates. Disadvantage of this deconjugation is that valuable information about the steroid profile in the sample is lost. In this study we developed a method to analyze steroids at very low concentration levels (ng.L⁻¹) for the free steroid, glucuronide and sulphate conjugates in urine samples. This method was used to determine concentrations of natural (pro)hormones in a large population (n=620) of samples from male and female bovine animals and from bovine animals treated with testosterone-cypionate, estradiol-benzoate, dihydroepiandrosterone and pregnenolone. The data acquired in this study was used to build a statistical model applying the multivariate technique 'Soft Independent Modeling of Class Analogy' (SIMCA). It is demonstrated that using this model, the results of the urine analysis can indicate for which animal's illegal treatment with natural (pro)hormones may have occurred.</p>

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3 1 **Endogenous steroid profiling by gas-chromatography tandem mass**
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5 2 **spectrometry and multivariate statistics for detection of natural hormone**
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7 3 **abuse in cattle**
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12 5 M.H. Blokland¹, E.F. van Tricht¹, H.J. van Rossum¹, S.S. Sterk¹ and M.W.F. Nielen^{1,2}
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23 10 the Netherlands

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26
27 11 **Abstract**

28 12 For years it is suspected that natural hormones are illegally used as growth promoters in cattle
29 13 in the EU. Unfortunately there is a lack of methods and criteria that can be used to detect the
30 14 abuse of natural hormones and distinguish treated from non-treated animals. Pattern recognition
31 15 of steroid profiles is a promising approach for tracing/detecting the abuse of natural hormones
32 16 administered to cattle. Traditionally steroids are analyzed in urine as free steroid after
33 17 deconjugation of the glucuronide (and sulphate) conjugates. The disadvantage of this
34 18 deconjugation is that valuable information about the steroid profile in the sample is lost. In this
35 19 study we developed a method to analyze steroids at very low concentration levels (ng.L⁻¹) for
36 20 the free steroid, glucuronide and sulphate conjugates in urine samples. This method was used to
37 21 determine concentrations of natural (pro)hormones in a large population (n=620) of samples
38 22 from male and female bovine animals and from bovine animals treated with testosterone-
39 23 cypionate, estradiol-benzoate, dihydroepiandrosterone and pregnenolone. The data acquired in
40 24 this study was used to build a statistical model applying the multivariate technique ‘Soft
41 25 Independent Modeling of Class Analogy’ (SIMCA). It is demonstrated that using this model,
42 26 the results of the urine analysis can indicate for which animal’s illegal treatment with natural
43 27 (pro)hormones may have occurred.

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50 28 **Keywords:** natural hormones, abuse, cattle, gas-chromatography tandem mass spectrometry
51 29 and multivariate statistics
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31 **Introduction**

32 It is known (Costain, et al. 2008; Nielen, et al. 2001) for years that natural hormones are
33 illegally administered to cattle to promote rapid growth and increase the feed conversion rate.
34 Several natural hormones were identified in illegal (injection) preparations found at farms.
35 Abuse of these compounds to promote growth in livestock is prohibited within the EU as laid
36 down in Directive 96/22/EC (Directive) and 2003/74/EC (Nielen, Vissers, et al. 2001).
37 However, control on abuse of natural hormones is very difficult due to the background levels
38 naturally present in livestock. Currently there are two methods available to detect natural
39 hormones in livestock. One technique is isotope ratio mass spectrometry (IRMS) (Buisson,
40 et al. 2005; Hebestreit, et al. 2006). This technique can discriminate synthetic hormones from
41 natural hormones in samples of urine. This method is very powerful and promising, although
42 it is limited to only two natural hormones so far; testosterone and estradiol. Disadvantages of
43 this technique are, that it is not very sensitive, and that labor intensive clean-up is necessary
44 and thus using this approach is rather expensive and time consuming. Another approach is the
45 analysis of steroid-esters in hair samples (Aqaiet al. 2009; Nielen et al. 2006; Stolker et al.
46 2009). That method works well when steroid-esters are administered. Potential drawbacks of
47 this method are that external contamination of hair samples cannot be fully excluded and that
48 the mechanism of deposition in the hair is not known in full detail yet.

49 Natural hormones are metabolized by the liver or intestine (phase I metabolism). Then
50 steroids are converted to more polar compounds by conjugation in order to be excreted via
51 urine from the body (phase II, metabolism). Circulating natural hormone levels are
52 maintained by a dynamic system that ensures homeostasis. In simplest terms, this system can
53 be considered a balancing act between the rate of synthesis and the rate of its metabolic
54 inactivation/elimination. As this system is in balance, both the rate of synthesis and the rate

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3 55 of metabolic inactivation/elimination have impact on the circulating levels of natural
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5 56 hormones. Chemically induced disruptions in either of these two processes can potentially
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7 57 modify circulating natural hormones levels. From a theoretical point of view, administration
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9 58 of natural hormones will disrupt this balance and will change the excretion patterns.
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11 59 Therefore, knowledge of the concentrations (Nielen et al. 2007) and the mutual relation of
12
13 60 these compounds in urine is important. The mutual relation is determined in this study via
14
15 61 metabolic profiling. Changes in these patterns can possibly be used as screening tools in
16
17 62 control schemes (Mareck et al. 2008). Metabolic profiling is targeted analysis of metabolites
18
19 63 and intermediates in a (part of a) biological system, typically large datasets are being
20
21 64 acquired. To handle these datasets dedicated statistical tools based on multivariate techniques
22
23 65 are applied, such as Principle Component Analysis (PCA), to elucidate differences between
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25 66 samples.
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29
30 67 The concept of the use of metabolic profiling to detect the illegal use of substances
31
32 68 originates from human sports doping research where the testosterone/epitestosterone ratio
33
34 69 (T/E ratio) (Donike et al. 1983) is applied to demonstrate the abuse of testosterone. An
35
36 70 extension of the T/E ratio in human sport doping research has recently been proposed by
37
38 71 Hemmerbach et al. (1994) and more recently by van Renterghem et. al (2010a). More ratios
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40 72 of metabolites from the natural metabolic pathway were included to build a statistical model
41
42 73 to determine dihydrotestosterone (DHT) and (dehydroepiandrosterone) DHEA misuse in
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44 74 sports. For several metabolites reference concentration values in urine are proposed to
45
46 75 determine if natural steroids are misused (van Renterghem et al. 2010b).
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49
50 76 The feasibility of T/E ratio to determine if synthetic 17β -testosterone is administrated
51
52 77 to cattle was tested by Angeletti et al., (2006). Due to the higher conversion rate of
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54 78 testosterone to epitestosterone in cattle this ratio cannot be used. A limited number of
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56 79 scientific papers is available covering the use of metabolic profiling to detect abuse of natural
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3 80 hormones in livestock farming. An approach similar to the testosterone/epitestosterone ratio
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5 81 in cattle was introduced by Fritsche et. al. (1998) to distinguish between beef from bulls and
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7 82 beef from steers. They used the masculinity index (MI) which is calculated from testosterone,
8
9 83 epitestosterone, and pregnenolone concentrations in meat samples. By means of the MI, a
10
11 84 classification of a beef sample of unknown origin was performed, regardless of the slaughter
12
13 85 age of the animal. However, the MI cannot be used to indicate abuse of natural hormones. Le
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15 86 Bizec et. al (2006) described the measurement of natural occurring steroids in kidney samples
16
17 87 before and after treatment with natural hormones. By applying discriminant statistics they
18
19 88 were able to separate the animals treated with natural hormones from the non-treated animals.
20
21 89 A similar approach was developed by Scarth et. al.(2010; 2011) , by measuring
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23 90 concentrations of natural hormones and markers in urine from cattle that was not treated with
24
25 91 natural hormones. As indicated in the reviews of Scarth et. al. (Scarth et al. 2009) and
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27 92 Mooney et. al. (2008) important topics in the field of steroid abuse are e.g. validation of the
28
29 93 methods and introduction of high throughput analysis. Furthermore they suggested that more
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31 94 research should be conducted on the applicability of multivariate statistical approaches to
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33 95 detect steroid abuse in cattle.
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38 96 Aim of the present study was to develop a urine analysis method that is capable of
39
40 97 discriminating between animals treated with natural hormones and untreated animals. The
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42 98 sample clean-up method developed in this study was based on previously (Tricht et al. 2008)
43
44 99 developed method which analyzes natural hormones in serum. Detection of the individual
45
46 100 compounds is based on gas-chromatography tandem mass spectrometry (GC-MS/MS),
47
48 101 covering the steroidogenesis of almost all major natural (pro)hormones, as depicted in Figure
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55 103 (figure 1)
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2
3 105 A large number of urine samples – collected at farms in the Netherlands and from
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5 106 cattle treated with natural hormones – were analyzed using this method. The data collected
6
7 107 were used to build a statistical model that can identify possible abuse of natural hormones.
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9

10 11 **Materials and methods**

12 13 14 15 ***Bovine urine samples collected at farms***

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17 110 Bovine urine samples were obtained from Dutch routine control programs in the period of
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19 111 2008 and 2009. Information on the animals such as age and gender was registered, but the
20
21 112 origin of the samples was kept anonymous. Since the samples were collected at farms all over
22
23 113 the Netherland it cannot be guaranteed that all were from non-treated animals. Since the
24
25 114 population is large and the average rate of animals testing positive in EU monitoring
26
27 115 programs is low the assumption that most of the samples are from animals which were not
28
29 116 treated with any growth promotors is justified. After sample collection the urine samples
30
31 117 were stored at -20°C until analysis. In total 620 urine samples were collected in order to
32
33 118 obtain a sufficiently large population to perform a reliable statistical evaluation. From the 620
34
35 119 samples 510 were used to build the assumed untreated reference collection, the others 110
36
37 120 were divided over the different animal experiments; estradiol-benzoate n=35 testosterone-
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39 121 cypionate n=14, DHEA n=29, pregnenolone n=32.
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45 46 47 ***Controlled animal treatment experiments***

48 123 Urine samples from different animal studies were used. The studies were performed
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50 124 following ethical approval at either CVI, Lelystad, the Netherlands or University of Ghent,
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52 125 Belgium.
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126 *Estradiol-benzoate treatment*

127 In this experiment a heifer was injected twice intra-muscularly in the neck with estradiol-
128 benzoate. The first injection was performed at the beginning of the treatment period; the
129 second injection one week after the first injection. The injection solution was prepared by
130 dissolving 248 mg in 2 ml of acetone, after which 50 ml of olive oil was added. The acetone
131 was evaporated under a stream of nitrogen. At t=0 and t=5 days a portion of 25 ml of the
132 solution was injected in the neck. Urine samples were collected prior to injection and at each
133 day after injection for a period of 21 days. The collected samples of urine were stored at –
134 20°C until analysis.

135 *Testosterone-cypionate treatment*

136 In this experiment a heifer was injected twice intra-muscularly in the neck with β -
137 testosterone-cypionate. The first injection was performed at the beginning of treatment
138 period; the second injection one week after the first injection. The injection solution was
139 prepared by dissolving 300 mg in 2 ml of acetone, after which 50 ml of olive oil was added.
140 The acetone was evaporated under a stream of nitrogen. A portion of 25 ml of olive oil was
141 injected in the neck. Urine samples were collected prior to injection and at each day after
142 injection. The collected samples of urine were stored at –20°C until analysis.

143 *DHEA-treatment*

144 In a time span of one and a half year, three independent bovine DHEA treatment experiments
145 were performed using identical treatment and sampling schedules. Male Friesian bovines
146 were purchased at the local market and housed for 2–3 weeks before the start of each
147 experiment. Each of the three experiments consisted of two animals of which one was treated
148 orally by capsules containing 1000 mg of DHEA, and the other intramuscular by 1000 mg of

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3 149 DHEA dissolved in 10 mL of Miglyol 812. Repeated dose administrations were performed
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5 150 seven times at 24h intervals. Before the start of the treatment urine collections were made,
6
7 151 and during the animal trials urine was sampled at days 2, 5, and 7. Untreated control animals
8
9 152 were included in all three experiments; three animals in the first experiment, one in the
10
11 153 second and two in the third experiment.

154 *Pregnenolone-treatment*

155 The pregnenolone experiment consisted of four male control animals and four male animals
156 which were treated orally with capsules containing 500 mg of pregnenolone. Repeated dose
157 administrations were performed seven times at 24h intervals. Before the start of the treatment
158 urine was collected and during the animal trials urine was sampled at days 2, 5, and 7.

159 *Determination of specific density and total solid content*

160 Refractometry is a relatively simple method to determine the total amount of solids in urine
161 (Weeth, H et al. 1969). Using the specific density of the samples, inter-sample variability in
162 the measured concentration caused by differences in density can be corrected. Correction was
163 performed using the average value 1.020 for the specific gravity of all bovine urine samples
164 used during this study.

$$\text{Concentration} = \frac{1.020 - 1}{\text{Specific Density Sample} - 1} \times \text{Concentration Sample}$$

165 Samples with a specific density lower than 1.004 were rejected for data-processing since the
166 correction factors for these samples are too large and the measurement of the specific density
167 is less reliable below 1.004.

168 *Materials*

169 Standard solutions of 1 ug l⁻¹ the analytes (table 1) were prepared in ethanol. Thousand- and

1
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3 170 ten thousand-fold dilutions of the standards were made and stored at -20°C until analysis.
4
5 171 Methanol and ethanol were obtained from (Biosolve, the Netherlands). Acetone, acetonitrile,
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7 172 ethanol, n-pentane, iso-octane, dry ethyl acetate and Tris(hydroxymethyl)-amino-methane
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10 173 were of analytical grade and obtained from Merck (MO, USA). N-Methyl-N-trimethylsilyl-
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12 174 trifluor(o)acetamide (MSTFA) was obtained from Alltech (IL, USA), ammonium iodide was
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14 175 obtained from Fluka (MO, USA), dithiothreitol was obtained from AnalaR (IL, USA). β -
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16 176 Glucuronidase was obtained from Roche diagnostics (Switzerland). Derivatization reagent
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18 177 (MSTFA++) consisted of N-methyl-N-trimethylsilyltrifluoroacetamide/ammonium
19
20 178 iodide/dithiothreitol (1000:2:4, v/w/w). Demineralized water was obtained from a milli-Q
21
22 179 purification system. Bond Elut C18 500 mg (3ml) SPE columns were obtained from Varian
23
24 180 (CA, USA). Set of guaranteed blank bovine reference urines, bov01 to bov20 (Sterk, S et al.
25
26 181 1998), was obtained from EURL for residues (the Netherlands).
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31 ***GC-MS/MS analysis***

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33 183 A Varian 1200L triple quadrupole mass spectrometer equipped with a CP8400 autosampler
34
35 184 and a CP-3800 GC was used. The GC column was a VF-17MS (L = 30 m, id = 0.25 mm, df =
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37 185 0.25 μ m), obtained from Varian. Two microliter of the purified samples or standard solutions
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39 186 was splitless injected onto the GC column at a pulsed pressure of 30 psi. The temperature
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41 187 program started at 110°C (constant for 1 min), increased 20°C.min⁻¹ to 240°C and was held
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43 188 for 1.5 min. Then, the temperature was increased 1°C.min⁻¹ to 244°C. Finally, the
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45 189 temperature was increased 25°C.min⁻¹ to 340°C. This temperature was held for 2 min. The
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47 190 helium flow was kept constant at 1.0 ml.min⁻¹. The GC-MS/MS was operated in electron
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49 191 ionization (EI) mode using Multiple Reaction Monitoring (MRM). In Table 1 the ion
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51 192 transitions monitored and the applied collision energy for each ion transitions is shown for all
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53 193 compounds analyzed.
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194 ***Analytical procedure***195 *Solid phase and liquid-liquid extraction*

196 To 2 ml of urine 1 ml of water was added and next 5 ng of internal standard mixture by
197 adding 50 µl of 0.1 ng µl⁻¹ internal standard mixture. The samples were loaded onto a
198 preconditioned (3 ml of methanol and 3 ml of milli-Q water) C₁₈ disposable solid phase
199 extraction (SPE) column. The SPE C₁₈ column was washed with 3 ml of milli-Q water. The
200 conjugated compounds were eluted with 3 ml 35/65 (%-v/v) acetonitrile/water; this eluate
201 was denoted glucuronide and sulphate fraction (G+S) and further processed as described in
202 the paragraph deconjugation and isolation of glucuronide conjugates. Next, the unconjugated
203 steroids (steroid aglycons) were eluted with 3 ml of acetone; that fraction contained steroid
204 steroid aglycons and was denoted A. Eluate A was evaporated at 50°C under a gentle stream
205 of nitrogen until nearly dry and re-dissolved in 100 µl of methanol and 2 ml of TRIS-buffer at
206 pH 9.2. Liquid Liquid Extraction (LLE) was performed twice with 7 ml of n-pentane. The
207 mixture was centrifuged for 5 minutes at 3000 g. The organic layer was collected in a glass
208 tube and evaporated to dryness at 55°C under a gentle stream of nitrogen and further
209 processed as described in the paragraph derivatization.

210 *Deconjugation and isolation of glucuronide conjugates*

211 The G + S fraction was dried at 55°C under a gentle stream of nitrogen and dissolved in 1 mL
212 of phosphate buffer (pH 7.4). 5 ng of internal standard mixture (see table 1) and 10 µL of β-
213 glucuronidase was added. The mixture was vortexed and hydrolyzed for three hours at 55°C.
214 An SPE C₁₈ column (3 mL) was preconditioned with 3 mL of methanol followed by 3 mL of
215 water. After that, 2 ml water was added to the SPE column and the hydrolyzed sample was
216 applied. The column was washed with 3 mL water. The sulphate-conjugates were eluted with
217 3 mL of 35:65 (v/v, %) acetonitril:water. The eluate collected, which was denoted fraction S,

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3 218 was evaporated to dryness at 55°C under a gentle stream of nitrogen and further processed as
4
5 219 described in paragraph deconjugation of sulphate conjugates. The deconjugated compounds
6
7 220 were eluted with 3 mL of acetone; this fraction was denoted G. The eluate was evaporated to
8
9 221 dryness at 55°C under a gentle stream of nitrogen and further processed as described in the
10
11 222 paragraph derivatization.

12 13 14 15 223 *Deconjugation of sulphate conjugates*

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18 224 To the dried extract (fraction S) 5 ng of internal standard mixture and 1 mL of dry ethyl
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20 225 acetate and 2 µL 4M H₂SO₄ were added. The solvolysis was performed for 1 hour at 40 °C
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22 226 after which 4 mL of ethyl acetate and 1 mL of an aqueous 5% NaHCO₃ solution were added.
23
24 227 The mixture was vortexed and centrifuged. The water phase was removed. The ethyl acetate
25
26 228 layer was transferred to a clean glass tube and evaporated to dryness at 55°C under a gentle
27
28 229 stream of nitrogen. The residue was dissolved in 100 µl of methanol and 2 mL of TRIS buffer
29
30 230 pH 9.2 was added. LLE was performed twice with 7 ml n-pentane. The pentane fraction was
31
32 231 evaporated to dryness at 55 °C under a gentle stream of nitrogen and further processed as
33
34 232 described in the paragraph derivatization.

35 36 37 38 39 233 *Derivatization*

40
41
42 234 The dried residues (fraction A, G and S) were derivatized separately. First they were
43
44 235 dissolved in 0.5 ml of ethanol and transferred into a derivatization-vial and evaporated to
45
46 236 dryness at 50°C under a gentle stream of nitrogen. The dry residue was derivatized by adding
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48 237 25 µl of MSTFA++ followed by incubation of 1 hour at 60°C. The derivatized mixture was
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50 238 evaporated to dryness at 50°C under nitrogen. The dry residue was reconstituted in 25 µl of
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52 239 iso-octane.

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9 243 ***Validation of the method***

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11 244 Validation was performed for all compounds in Table 1. For these compounds the $CC\alpha$, $CC\beta$

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13 245 and measurement uncertainty were determined. The decision limit ($CC\alpha$) and detection

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15 246 capability ($CC\beta$) (2002/657/EC) were determined using a three point standard calibration

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17 247 curve in different blank urine samples. Each individual sample was analyzed six-fold on each

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19 248 day. The measurement was repeated on three different days. From this calibration curve the

20
21 249 y-intercept and slope were calculated ($y=ax+b$). $CC\alpha$ is the corresponding concentration at

22
23 250 the y-intercept plus 2.33 times the standard deviation of the y-intercept ($\alpha=1\%$). The

24
25 251 calculated $CC\alpha$ was checked by spiking samples at the corresponding levels. The $CC\beta$ is the

26
27 252 corresponding concentration at the decision limit plus 1.64 times the standard deviation of the

28
29 253 y-intercept ($\beta=5\%$). The (within day) repeatability and within laboratory reproducibility

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31 254 (between days) were determined from this dataset using the approach as described in ISO

32
33 255 5725 (ISO 5725, 1994). To determine the measurement uncertainty all variances were

34
35 256 summed. For this validation the variances are equal to the within days reproducibility. The

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37 257 measurement uncertainty at the different spiking levels is calculated with the equation:

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39 258 measurement uncertainty $U = 2S_R$, with S_R being the sum of the variances. All the variances

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41 259 are expressed as a coefficient of variance.
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49 260 ***Data Processing***

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51 261 GC-MS/MS Data was automatically processed using MS Workstation[®] software from

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53 262 Varian. All peak integrations were manually checked and baseline corrected if necessary.

54
55 263 Concentrations below the $CC\alpha$ were rejected and not used for the statistical evaluation of the
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3 264 results. If the measured concentration was outside the calibration range the sample was re-
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5 265 analyzed following dilution.
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9 266 ***Statistical Analysis of the data***

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11 267 Multivariate analyses were performed using Pirouette version 4.0 from Infometrix[®] (Bothell,
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13 268 Washington, USA) The data was first explored using Principle Component Analysis (PCA),
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15 269 showing separation between all groups used in this study, e.g. blank bovine female and male,
16
17 270 testosterone, estradiol, DHEA and pregnenolone treatment. However, PCA is an
18
19 271 unsupervised model and better suited supervised statistical models are available to perform
20
21 272 pattern recognition. A start was made with a number of objects whose group membership is
22
23 273 known, for example testosterone treated animals. These objects were considered the
24
25 274 'learning' or 'training' objects (Miller, J et al. 2005). The aim of supervised pattern
26
27 275 recognition methods is to use these objects to find a rule for allocating new objects of the
28
29 276 unknown group to the correct group. A number of supervised statistical models were
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31 277 explored. K-nearest neighbor method, (linear) discriminant analysis and SIMCA. All data
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33 278 was mean-centered and log₁₀ transformed to suppress exorbitant values.
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40 279 **Results and Discussion**

41
42 280 ***Validation of the method***

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45 281 The described method was validated as a quantitative confirmatory method according to
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47 282 Commission Decision 2002/657/EC (2002/657/EC, CD 2002). The analyses were performed
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49 283 in the same way as routine analyses of unknown samples, with the addition of internal
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51 284 standards. Because sulphate and glucuronide standards are in contrast with steroid steroid
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53 285 aglycons hardly commercially available it is not possible to fully validate the method for
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55 286 these conjugates. As samples are generally cleaner after the first SPE step, it is expected that
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3 287 the $CC\alpha$ and $CC\beta$ are in the same range as they would be for the steroid steroid aglycons.
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5 288 Therefore the same $CC\alpha$ and $CC\beta$ are used in this study for the steroid steroid aglycons and
6
7 289 conjugated compounds. Several performance characteristics were determined. Important
8
9 290 parameters are the decision limit ($CC\alpha$), the limit at and above which it can be concluded
10
11 291 with an error probability of α that a sample is non-compliant, and the detection capability
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13 292 ($CC\beta$), the smallest content of the substance that may be detected, identified and/or quantified
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15 293 in a sample with an error probability of β . In the case of substances for which no permitted
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17 294 limit has been established, the detection capability is the lowest concentration at which a
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19 295 method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$. In
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21 296 Table 2 an overview is given of the validation results.
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27
28 298 (table 2)

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31 300 The results of the validation confirm that GC-MS/MS measurement of a large group of
32
33 301 (pro)hormones is feasible. For all natural steroids measured the analytical parameters are
34
35 302 typical for methods used for residue analysis in bovine urine with $CC\alpha$ for all compounds in
36
37 303 the range of 0.007-0.46 ng.ml⁻¹. The measurement of uncertainty is for most compounds
38
39 304 relative high. There are two main reasons for this, all measured compounds occur
40
41 305 endogenous in samples of urine which of course attributes to the variation, and it can be
42
43 306 expected that the variability is relative high because the developed method is capable to
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45 307 measure steroids at very low pg.ml⁻¹ levels. It is difficult to compare the validation results
46
47 308 with two recent studies (Regal et al. 2009; Scarth et al. 2010) of comparable goal and
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49 309 methods. These studies different by matrix, serum instead of urine, and the validation was not
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51 310 performed according to Commission Decision 2002/657(2002).
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3 311 ***Quantitative urine analysis***
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5 312 In total, almost 620 samples were analyzed for the compounds given in Table 2. For all
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7 313 compounds the concentrations of the steroid aglycon, glucuronide and sulphate fractions were
8
9 314 determined. See for the average values and the 95% Confidence Interval (CI) for the male
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11 315 and female population, and for the treated animals table 3.
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18 317 (table 3)
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22 319 In theory, treatment with natural hormones should change the mutual relations and
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24 320 therefore influence the typical concentrations of steroids involved in the steroidogenesis. To
25
26 321 visualize the effects of administration of natural hormones on their concentrations in urine
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28 322 and to explore the data as depicted in table 3, heatmap plots (figure 2 and 3) were
29
30 323 constructed. Treatment of female and male animals with different natural steroids and the
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32 324 average concentration values of all male and female animals from the reference population
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34 325 are depicted in figures 2 and 3.
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40 327 (figure 2)
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46 329 ***Testosterone treatment (female bovine)***
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48
49 330 The heatmap in figure 2 gives a quick overview of the increase in concentrations of different
50
51 331 natural hormones after treatment. The concentrations of the steroid aglycon, glucuronide and
52
53 332 sulphate of 17β -testosterone and its major metabolite 17α -testosterone are increased and
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55 333 slowly decrease over time. Possibly because the levels of testosterone-cypionate in the
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57 334 injection depots decreases. Most of the testosterone is rapidly metabolized (figure 1) to the
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3 335 steroid aglycon and glucuronide of ethiocholanolone; the steroid aglycon, glucuronide and
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5 336 sulphate of 5α -androstanediol- $3\beta,17\alpha$; and androstenedione-sulphate. These effects can be
6
7 337 expected since this is the metabolism pathway of testosterone. When the heatmap is further
8
9 338 examined some other effects are observed. There is an increase in the levels of all three forms
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11 339 of DHEA and especially the steroid aglycon form of DHEA. This is not expected since
12
13 340 DHEA is a precursor of testosterone and not a metabolite. Furthermore there is an increase in
14
15 341 the steroid aglycon form of androstenediol and pregnenolone. This suggests some sort of
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17 342 feedback mechanism, which to the best of our knowledge, was not observed in other studies.
18
19 343 A possible explanation is that the biosynthesis from androstanediol to testosterone is inhibited
20
21 344 due to the high exogenous level of testosterone, inducing an overproduction of all steroids
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23 345 involved in this pathway (see Figure 1), immediately following injection of testosterone-
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25 346 cypionate. This would also increase the production of the hormone DHEA-sulphate. We do
26
27 347 not know if this effect is limited to the particular animal used in this study or that these
28
29 348 effects are generic in female bovine animals treated with testosterone-cypionate. To
30
31 349 determine the excretion and feedback routes of testosterone administration on DHEA and
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33 350 other steroids more experiments are needed. The following experiments could be performed;
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35 351 labeling ($2H$, $13C$) of administrated steroids, the use of IRMS, however, low concentrations
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37 352 will limit the use of IRMS, or the use of ^{14}C labeling and analysis by Accelerator Mass
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39 353 Spectrometry (AMS).

354 *Estradiol treatment (female bovine)*

355 Treatment of a female bovine animal with 17β -estradiol-3-benzoate showed less pronounced
356 effects when the heat-map (figure 2) is examined. There is an increase in the concentrations
357 of the steroid aglycon, glucuronide and sulphate of 17β -estradiol and its major metabolite
358 17α -estradiol. Estradiol itself is metabolized to estrone (figure 1). Therefore, an increase in

1
2
3 359 the concentration of estrone-glucuronide is observed. The levels of estradiol and estrone
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5 360 decrease over time possibly because the concentration of estradiol-benzoate in the injection
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7 361 depot decreases over time. Less pronounced is the increased production of testosterone-
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10 362 sulphate, 5α -androstenediol- $3\beta,17\alpha$ -glucuronide and 5α -androstenediol- $3\alpha,17\alpha$ -sulphate
11
12 363 indicating possible feedback of testosterone via this route because the route to estradiol is
13
14 364 inhibited due to the increased concentration of exogenous 17α -estradiol. Since these results
15
16 365 are from the treatment of only one animal no general conclusions can be made on the
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18 366 metabolism pattern for estradiol-benzoate treatment. However, this treatment again
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21 367 demonstrates that besides the concentrations changes of other compounds, the administered
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23 368 natural hormone and its metabolites change as well, in accordance with expectations.
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28 370 (figure 3)

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32 372 ***DHEA treatment (male bovine)***

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35 373 After treatment with DHEA there is an increase in the concentrations of the steroid aglycon,
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37 374 glucuronide and sulphate for the following compounds: DHEA, androstenediol, α -
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39 375 testosterone and 5α -androstenediol- $3\beta,17\alpha$. There also is a slight increase in concentration of
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41 376 some other metabolites, e.g: androsterone-sulphate, ethiocholanolone-glucuronide and 5α -
42
43 377 androstenediol- $3\alpha,17\alpha$ -glucuronide. All these steroids are metabolites from DHEA and
44
45 378 clearly demonstrate the clearance of DHEA after treatment. Some of the detected metabolites
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47 379 were previously suggested as DHEA metabolites in an LC-ToF-MS study (Rijk et al. 2009).

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51 380 Less pronounced, but also observed in this treatment is the feedback towards
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53 381 precursors of DHEA: there is a slight increase in concentration of pregnenolone-glucuronide
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55 382 and pregnenolone-sulphate. These effects are observed in all animals included in this study,
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57 383 some animals react stronger than others on the treatment; this difference is probably caused
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3 384 by the type of treatment, intramuscular or oral, and/or on the metabolism rate of the particular
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5 385 animal.
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9 386 ***Pregnenolone treatment (male bovine)***

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11 387 The effect of treatment of the male bovine animals with pregnenolone is less pronounced than
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13 388 other treatments studied. An increase in the concentrations of the steroid aglycon,
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15 389 glucuronide and sulphate of pregnenolone is observed, and there also seems to be a minor
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17 390 effect on the steroid aglycons of progesterone and DHEA. This increase in concentration of
18
19 391 these steroid aglycons can be explained by the metabolic route of pregnenolone. Although the
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21 392 changes in hormone concentrations are less clear than for the previously described
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23 393 treatments, subtle changes are observed in the metabolism pattern after treatment with
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25 394 pregnenolone.
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33 396 ***Multivariate statistics of the data***

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36 397 It is known from other studies (Rijk et al. 2009) that biomarkers can be identified for certain
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38 398 treatments with growth hormones. Some biomarkers are considered quite unique, e.g. 19-
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40 399 noretiochlanolone (Scarth et al. 2010) in pigs, 5 α -estrane-3 β ,17 α -diol (Pinel et al. 2010) for
41
42 400 treatment of nortestosterone in bovine. For treatment with boldenone 6 β -hydroxy-17 α / β -
43
44 401 boldenone (Blokland et al. 2007) was as unique marker identified. Such unique markers are,
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46 402 so far, not found for treatment with natural hormones. It is indicated in literature that after
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48 403 natural hormone treatment, the concentration of certain hormones that are part of the normal
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50 404 steroidogenesis are increased relative to the concentration levels before treatment. This study
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52 405 confirms that the concentration of natural hormones involved in the steroidogenesis are up-
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54 406 regulated after treatment with natural hormones. The effect depends on the type of natural
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3 407 steroid administered. Apart from increased concentrations of the steroids administered and
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5 408 up-regulation of related steroids, concentration changes of precursors of the steroids
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7 409 administered were observed. By combining all observed effects on the concentration and by
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9 410 applying advanced statistics, it should be possible to discriminate between treated and
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11 411 untreated animals.

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14 412 The reference population consists of urine samples obtained from routine control
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16 413 programs in The Netherlands which were collected from bovine animals of all ages and both
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18 414 genders. To assess the effects of natural hormone administration, animals treated with
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20 415 estradiol-benzoate, testosterone-cypionate, DHEA and pregnenolone were also included in
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22 416 the sample set. The whole data set was processed using Soft Independent Modeling of Class
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24 417 Analogy (SIMCA) software. SIMCA produced the best results for the data collected in this
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26 418 study in terms of prediction power and description. SIMCA first models data within each
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28 419 class (e.g. male, female, testosterone treatment etc.) using Principle Component analysis
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30 420 (PCA). Each group is modeled based on similarities within a group. Therefore, the number of
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32 421 principle components will vary per group. After defining the optimum settings for each
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34 422 group, the whole data set was used to separate the groups and build the statistical model.
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36 423 Then, samples could be classified by determining their distance to and degree of resemblance
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38 424 with each group. An advantage of SIMCA is that it can indicate with a certain degree of
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40 425 probability that a sample belongs to one or more groups, or to none of the groups used to
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42 426 build the model. The SIMCA model was optimized to maximize the correct classification
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44 427 rate. The concentrations were log₁₀ transformed and mean-centered, maximum factors 6,
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46 428 scope local, probability of the statistics used was 0.95. For each group the number of selected
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48 429 factors was optimized. Increasing the number of factors was ceased when the loadings
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50 430 became too noisy and therefore did not add any new information to the model, but made it
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3 431 less reliable. The final model including all data can be reviewed visually (limited to three
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5 432 dimensions) using class projection (figure 4).
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10 434 (figure 4)

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15 436 From the class projection of the SIMCA analysis (fig. 4), several observations can be made.

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17 437 There are differences between the groups, especially between the treated and non-treated

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19 438 animals. All groups are separated to a certain extent requiring the first three principle

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21 439 components. This separation indicates that the model can be used to predict which group

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23 440 unknown samples belong to. Since the groups are not perfectly separated, the classification

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25 441 will most likely not be perfect for all samples. In particular for the separation of the male and

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27 442 female untreated groups. The predictive power of the model is described by the

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29 443 misclassification (or confusion) matrix. The misclassification matrix describes how the data

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31 444 used to build the model would fit on the model. Applying the constructed SIMCA model

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33 445 makes it possible to determine to which group a certain animal belongs; male, female or

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35 446 treated with compound X. A unique feature of SIMCA is that samples can also be classified

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37 447 as belonging to none of the groups, indicating an unknown metabolic pattern or treatment. If

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39 448 a sample is classified close to another group during classification it will be indicated as “next

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41 449 best”. This next best score has to be taken into account when samples are classified. A

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43 450 perfectly classified sample has for the next best score zero. The misclassification matrix of

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45 451 the data set is shown in table 4.

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48 452 (table 4)

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55 454 From table 4 it can be concluded that the prediction for treated animals is excellent. The

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57 455 scores of the male and female control group are not 100% correct. This can be explained by

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3 456 the fact that the female and male control group are less homogenous compared to the animals
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5 457 in the treated groups. Also are a few female samples classified in a treated group. This can be
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7 458 caused by natural variations in steroid profiles which causes a sample to be incorrectly
8
9 459 classified or by the fact that we cannot be sure that all samples of the reference population
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11 460 used were untreated. The steroid profiles of the male and female groups are influenced by
12
13 461 age, animal breed, and external influences such as e.g. food and housing. If the reference
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15 462 population would be smaller and more homogenous, the prediction values would have
16
17 463 definitely been better. However, the ultimate goal is to use the model for control programs.
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19 464 Therefore it is necessary to include more different treated and non-treated bovine animals in
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21 465 the model. It is not a major problem if the gender of an animal is not correctly assessed; it is
22
23 466 far more important that treated animals differentiate from a normal population of animals.
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25 467 With this model it is possible to classify treated animals correctly. There is a chance that the
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27 468 number of misclassifications in the treated animal population increases if more different
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29 469 treatments are included in the model. On the other hand, more samples from treated animals
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31 470 make it possible to further refine the model and therefore increase its predictive power.
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38 471 *Cross-validation of the model*

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40 472 The model was validated by means of cross-validation. Cross-validation is a variant of
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42 473 classification analysis where, particular cases are removed from the data set, the reduced data
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44 474 set is then used to classify the omitted cases as if it were new cases. For validation of the
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46 475 model, 10% of the samples were removed from the model. The model was then built as
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48 476 described before with 90% of the data and 10% removed samples were classified. In table 5
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50 477 an overview is given of this validation.
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56 479 (table 5)

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3 481 With the 10% holdout all samples from the treated animals could be successful classified
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5 482 correctly using the SIMCA model. The next best classification for DHEA and estradiol is
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7 483 corresponding to the correct gender. The next best classification for both pregnenolone and
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9 484 testosterone is DHEA or male. In the heat-map of the pregnenolone treatment (fig. 3) can be
10
11 485 seen that there is some increase in the steroid aglycon of DHEA and testosterone which could
12
13 486 explain the classification in the male (higher testosterone levels) and DHEA group. The next
14
15 487 best classification for testosterone is DHEA or male. This is less surprising, from the heatmap
16
17 488 [fig. 3] it can be observed that there is an increase in the steroid aglycon of DHEA resulting
18
19 489 in classification in the DHEA group. An increase in testosterone after treatment with this
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21 490 compound is shown resulting in classification in the male group. Classification of the male
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23 491 and female animals in the opposite sex group can be expected from the results of the
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25 492 misclassification matrix. An explanation can be the age of the animals. Young animals have
26
27 493 very low concentration in sex steroids.
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32 494 To further evaluate the model, twenty guaranteed blank bovine samples were
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34 495 analyzed and classified. The blank bovine sample set consists of different breeds, genders and
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36 496 ages. Using this set, the model was verified for its accuracy towards classification of samples
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38 497 which were not included while building the model. It is also important for control purposes to
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40 498 establish that the model has a low false positive score. The results of the validation with the
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42 499 blank sample set are shown in table 6.
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46 500 (table 6)
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50 502 The results of the classification of guaranteed blank bovine samples are satisfying for
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52 503 this type of model. Only one sample was not assigned to the correct group; in this case a
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54 504 mixture of bovine samples which is assigned to the pregnenolone group. Probably this is due
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56 505 the fact that mixed urine was used, resulting in a profile that correspond with a treated
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3 506 animal. All other samples were assigned correctly which indicates that the number of false
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5 507 positive samples in screening is low when the model is used in a routine control programs.
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8 9 508 **General discussion**

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11 509 In a recent study (Anizan et al. 2011a) the glucuronide and sulphate conjugate concentrations
12
13 510 were used to predict natural hormone abuse. In this study (Anizan et al. 2011a) the ratio of
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15 511 [sum of (Epiandrosterone-Glucuronide + Androsterone-Sulphate + Epiandrosterone-Sulphate
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17 512 + alpha-Testosterone-Glucuronide + Etiocholanolone-Glucuronide) over DHEA-Sulphate
18
19 513 was calculated to determine whether treatment with the natural hormones androstenedione
20
21 514 had occurred. In another study (Anizan et al. 2011b) glucuronide and sulphate metabolites
22
23 515 were detected using precursor ion scan acquisition mode. This approach was tested using
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25 516 urine from animals treated with 4-androstenedione. A statistical model was build that could
26
27 517 be used to detect abuse of 4-androstenedione. The basic idea, to measure the concentration of
28
29 518 glucuronides and sulphates steroids, is comparable to our study, however, its implementation
30
31 519 is different. The concentrations of steroid aglycons were in both studies (Anizan et al. 2011a;
32
33 520 Anizan et al. 2011b) not taken into account. It is important to monitor steroid aglycon
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35 521 concentrations because their concentrations will also increase in case an animal is treated
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37 522 with a natural hormone. The described methods furthermore lack performance characteristics.
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41 524 The use of ratios of steroids and prohormones to determine abuse seems promising; in
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43 525 human doping studies the use of ratios has been extensively examined by van Renthergem
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45 526 (2010a). For a large population the concentrations of steroids and precursors were
46
47 527 determined. Combinations of ratios were statistically compared to determine if abuse of
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49 528 natural hormones had occurred. It was concluded that the use of ratios of several metabolites
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51 529 could help the decision making to determine if abuse with natural hormones had occurred.
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3 530 (Angeletti et al., 2006). Another approach is the use of statistical multivariate analysis to
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5 531 determine abuse of natural hormones as was demonstrated by Regal (Regal et al. 2009). In
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7 532 this study a multivariate model based on orthogonal projections to latent structures (OPLS)
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9 533 was built, based on high resolution accurate mass spectra LC runs of treated and non-treated
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11 534 animals. The model was used to determine what type of application form of estradiol was
12
13 535 used. The OPLS model was built on non-identified markers. This approach was used
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15 536 successful in this study but has to be further evaluated by adding more non-treated animals
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17 537 from control programs. However, this study demonstrates that statistical models can be
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19 538 predictive for the type of hormone treatment.
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540 **Conclusion**

541 With the analytical method developed in this study almost all major natural hormones present
542 in the steroidogenesis of bovine animals can be analyzed and classified. The concentration of
543 the glucuronide- and sulphate conjugates (phase II metabolites) can be quantitatively
544 determined. The analytical method was validated for natural hormones, according to
545 Commission Decision 2002/657/EC. Analytes can be detected in bovine urine at levels as low
546 as 7 ng.L⁻¹.

547 Treatment of bovine animals with natural steroids results in increased concentrations
548 of the steroid aglycon of administered compounds, as well as their glucuronide- and sulphate
549 conjugates. Furthermore, an increase in the concentration is observed for the compounds
550 involved in the major metabolic pathways of the administered compounds. In some cases
551 there is an increase in the glucuronide or sulphate conjugates of a metabolite related to the
552 excretion of the compounds. It was observed that there is a feedback mechanism resulting in
553 increasing concentrations of some of the precursors of the administered compounds. This was
554 rather surprising and to our knowledge not observed before. The obtained results were used

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3 555 to build a model based on SIMCA. The model was validated by means of a cross-validation
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5 556 and by analyzing a set of guaranteed blank urine samples of bovine animals. Validation
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7 557 indicated that the model can be used to classify animals in a treated and untreated group. We
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10 558 can conclude that the statistical model is a promising strategy to determine whether bovine
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12 559 animals are treated with natural hormones. This model can be used as a screening method to
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14 560 pinpoint suspect samples. Samples that are regarded as suspect, should be confirmed with,
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16 561 preferably, isotope ratio mass spectrometry or with other additional research.
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18 562 More data from different animals treated with natural hormones must be added to the
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20 563 database to make the model more robust. For use in routine monitoring programs, the model
21
22 564 needs simplifications, e.g. by omitting non-discriminative compounds from the GC-MS/MS
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24 565 quantitative analysis. Possibly the model can be used to investigate treatments with synthetic
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26 566 exogenous growth promoters such as stanozolol, since these also will influence the
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28 567 steroidogenesis and are known for feedback effects.
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37
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3 693 **Figure 1.** Steroidogenesis of compounds analyzed (steroid aglycons, glucuronide and
4 694 sulphate conjugates) in this study.

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8 696 **Figure 2.** Heatmap of the treatment of female bovine animals with estradiol-benzoate and
9 697 testosterone-cypionate. Average concentration calculated for 227 female bovine animals.
10 698 Samples are ranked from the first day of the treatment till the last day of the sampling period
11 699 after treatment. Dark squares are the highest concentrations and light colors the lowest
12 700 concentrations. Concentrations were \log_{10} transformed. Color map is equally distributed (16
13 701 steps) over all concentrations measured (A=steroid aglycon, G=Glucuronide-conjugate,
14 702 S=Sulphate-conjugate).

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18 704 **Figure 3.** Heatmap of the treatment of six different male bovine animals with DHEA, four
19 705 different male bovine animals with pregnenolone, samples of the blank control population,
20 706 and the average concentration for 226 male bovine animals. Dark green squares are the
21 707 highest concentrations, red squares the lowest. Concentrations were \log_{10} transformed. Color
22 708 map is equally distributed (16 steps) over all concentrations measured (A=steroid aglycon,
23 709 G=Glucuronide-conjugate, S=Sulphate-conjugate).

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27 711 **Figure 4.** Class projection of the SIMCA analysis of bovine male and female of the control
28 712 population, estradiol-benzoate, testosterone-cypionate, DHEA and pregnenolone treated
29 713 animals. See legend for type of sample the colors represent.

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716 **Table 1.** GC-MS/MS measurement conditions (CE=collision energy)

ID	Source	MRM1	CE (V)	MRM2	CE (V)
Pregnenolone	Searle	460 > 445	-8.0	460 > 157	-25.0
Progesterone	Steraloids	458 > 443	-7.5	458 > 157	-20.0
Progesterone- ¹³ C2	EURL	460 > 445	-8.0	-	-
DHEA	Steraloids	432 > 417	-7.5	432 > 327	-15.0
Androstenedione	Organon	430 > 415	-10.0	430 > 209	-12.5
Androstenediol	Sigma	434 > 344	-5.0	434 > 239	-25.0
17 β -Testosterone	Steraloids	432 > 301	-15.0	432 > 209	-12.5
17 α -Testosterone	Steraloids	432 > 327	-15.0	432 > 209	-12.5
17 β -Testosterone-d3	EURL	435 > 209	-13.0	-	-
Dihydrotestosterone	Sigma	434 > 405	-7.5	434 > 195	-22.5
Estrone	Steraloids	414 > 399	-7.5	414 > 155	-17.5
17 β -Estradiol	Diosynth	416 > 326	-7.5	416 > 285	-10.0
17 α -Estradiol	Organon	416 > 326	-7.5	416 > 285	-10.0
17 β -Estradiol-d3	EURL	419 > 285	-10.0	-	-
Etiocholanolone	Sigma	434 > 419	-10.0	434 > 329	-20.0
Androsterone	EURL	434 > 419	-10.0	434 > 329	-20.0
5 α -Androstenediol-3 β -17 β	Steraloids	436 > 241	-15.0	436 > 346	-5.0
5 α -Androstenediol-3 β -17 α	Steraloids	436 > 241	-15.0	436 > 346	-5.0
5 α -Androstenediol-3 α -17 β	Steraloids	436 > 241	-15.0	436 > 346	-5.0
5 α -Androstenediol-3 α -17 α	Steraloids	436 > 241	-15.0	436 > 346	-5.0

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719 **Table 2.** Overview of the validation results for the determination of steroids in bovine urine by GC-
 720 MS/MS, U = measurement of uncertainty (k=2)

ID	CC α (ng.ml ⁻¹)	CC β (ng.ml ⁻¹)	U (%)
Pregnenolone	0.18	0.30	68.7
Progesterone	0.10	0.17	24.1
DHEA	0.06	0.10	39.4
Androstenedione	0.12	0.20	88.0
Androstenediol	0.13	0.22	45.6
17β-Testosterone	0.03	0.04	14.9
17α-Testosterone	0.05	0.08	33.5
Dihydrotestosterone	0.10	0.18	71.3
Estrone	0.10	0.17	109
17β-Estradiol	0.007	0.01	11.4
17α-Estradiol	0.01	0.02	28.2
Etiocholanolone	0.06	0.10	46.0
Androsterone	0.06	0.11	69.8
5α-Androstenediol-3β, 17β	0.20	0.34	85.0
5α-Androstenediol-3β, 17α	0.27	0.46	90.7
5α-Androstenediol-3α, 17β	0.46	0.78	96.4
5α-Androstenediol-3α, 17α	0.26	0.45	57.8

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Table 3. Average concentration ($\mu\text{g}\cdot\text{L}^{-1}$) and CI (95%) of the steroid aglycon (A), glucuronide (G) and sulphate (S) for the female, male population and for the treated animals.

Compound	Conjugate	Female			Male			B-Estradiol-benzoate			B-Testosterone-cypionate			DHEA			Pregnenolone		
		Average	+95 CI	-95 CI	Average	+95 CI	-95 CI	Average	+95 CI	-95 CI	Average	+95 CI	-95 CI	Average	+95 CI	-95 CI	Average	+95 CI	-95 CI
5 α -Androstenediol-3 α ,17 α	A	0.26	0.26	0.26	0.32	0.25	0.40	0.26	0.26	0.26	0.34	0.26	0.41	2.31	-0.19	4.80	0.37	0.25	0.50
	G	3.96	0.97	6.96	2.39	1.82	2.96	0.26	0.26	0.27	1.50	0.74	2.26	63.83	34.00	93.66	0.29	0.23	0.35
	S	0.39	0.24	0.53	0.52	0.37	0.67	0.75	0.46	1.03	0.26	0.26	0.26	0.34	0.26	0.43	0.28	0.24	0.31
5 α -Androstenediol-3 α ,17 β	A	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.47	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
	G	0.53	0.43	0.62	0.47	0.46	0.48	0.46	0.46	0.46	0.46	0.46	0.46	0.47	0.45	0.49	0.46	0.46	0.46
	S	0.77	0.61	0.93	0.50	0.47	0.54	0.49	0.45	0.53	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
5 α -Androstenediol-3 β ,17 α	A	0.54	0.38	0.69	3.38	1.57	5.19	0.27	0.27	0.27	10.20	5.94	14.45	89.00	30.04	147.96	6.28	3.59	8.97
	G	95.74	75.17	116.32	125.26	109.28	141.23	9.07	6.20	11.95	46.22	25.79	66.65	2606.73	1933.31	3280.15	153.94	118.91	188.97
	S	2.09	1.44	2.75	3.21	2.55	3.88	0.99	0.63	1.35	2.32	1.62	3.02	96.16	55.85	136.46	5.46	2.58	8.33
5 α -Androstenediol-3 β ,17 β	A	0.20	0.20	0.20	0.22	0.18	0.26	0.20	0.20	0.20	0.20	0.20	0.20	2.25	0.43	4.06	0.31	0.15	0.47
	G	0.53	0.35	0.70	0.44	0.33	0.55	1.87	0.69	3.04	0.24	0.15	0.33	6.05	2.45	9.64	0.30	0.10	0.50
	S	0.85	0.37	1.32	1.94	1.20	2.69	1.08	0.71	1.44	0.20	0.20	0.20	3.76	1.13	6.38	0.48	0.32	0.65
Androstenediol	A	0.13	0.13	0.13	0.26	0.14	0.39	0.20	0.15	0.24	0.92	0.43	1.41	8.90	3.16	14.64	0.51	0.10	0.92
	G	7.97	6.02	9.92	13.95	12.38	15.52	0.53	0.13	1.19	0.25	0.05	0.46	36.08	23.38	48.77	2.37	1.63	3.12
	S	12.65	8.41	16.88	9.78	6.77	12.80	0.13	0.13	0.13	0.13	0.13	0.13	20.83	10.37	31.29	1.31	0.84	1.79
Androstenedione	A	0.23	0.18	0.27	0.49	0.27	0.70	0.12	0.12	0.12	0.49	0.12	0.86	5.08	1.26	8.91	0.41	0.33	0.48
	G	0.91	0.52	1.31	0.79	0.40	1.18	0.13	0.12	0.14	0.12	0.12	0.12	0.42	0.30	0.55	0.35	0.26	0.45
	S	0.32	0.16	0.47	0.20	0.14	0.25	0.12	0.12	0.12	1.39	0.90	1.88	1.32	0.40	2.24	0.12	0.12	0.12
Androsterone	A	0.06	0.06	0.07	0.10	0.07	0.13	0.06	0.06	0.06	0.07	0.06	0.09	3.99	0.06	9.85	0.07	0.06	0.08
	G	1.78	1.20	2.35	1.62	0.90	2.33	0.06	0.06	0.06	0.14	0.09	0.18	1.67	0.95	2.39	0.37	0.25	0.50
	S	0.11	0.06	0.16	0.08	0.06	0.11	0.06	0.06	0.06	0.06	0.06	0.07	1.73	0.95	2.50	0.08	0.06	0.10
DHEA	A	0.10	0.07	0.13	0.46	0.11	0.81	0.06	0.06	0.06	2.10	1.32	2.87	46.96	19.12	74.80	0.95	0.62	1.27
	G	10.07	8.54	11.59	7.35	6.48	8.21	1.82	1.35	2.28	5.83	2.91	8.74	694.05	445.67	942.44	18.86	13.98	23.74
	S	11.68	9.52	13.84	24.29	19.38	29.19	15.17	12.10	18.24	70.99	48.55	93.43	1182.18	737.59	1626.78	48.88	30.77	66.99
Dihydrotestosterone	A	0.11	0.10	0.13	0.12	0.10	0.14	0.66	0.34	0.99	0.10	0.10	0.10	0.16	0.10	0.23	0.10	0.10	0.10
	G	4.33	0.10	8.92	6.76	3.09	10.44	0.16	0.04	0.27	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.12
	S	0.18	0.10	0.25	0.47	0.22	0.73	0.52	0.02	1.03	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Estrone	A	0.11	0.10	0.12	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.10
	G	0.81	0.41	1.22	0.16	0.09	0.23	4.11	-0.59	8.80	0.10	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.10
	S	5.27	2.58	7.96	0.11	0.11	0.12	1.79	0.00	3.59	0.10	0.10	0.10	0.10	0.10	0.11	0.11	0.10	0.13
Etiocholanolone	A	0.11	0.07	0.14	1.67	0.06	3.48	0.06	0.06	0.06	10.09	1.31	18.86	164.31	0.06	403.85	6.97	3.90	10.04
	G	43.06	27.11	59.01	116.16	90.91	141.40	2.83	2.17	3.49	18.25	9.40	27.10	1228.59	258.70	2198.49	56.40	40.53	72.27
	S	0.42	0.18	0.66	0.92	0.51	1.33	0.14	0.06	0.31	0.06	0.06	0.06	1.89	0.62	3.17	0.19	0.06	0.40
Pregnenolone	A	0.20	0.19	0.22	0.22	0.19	0.24	0.18	0.18	0.18	0.65	0.46	0.84	0.26	0.18	0.33	15.16	8.20	22.11
	G	6.22	5.07	7.36	1.68	1.42	1.95	0.33	0.22	0.45	1.01	0.47	1.54	4.79	3.11	6.46	35.34	22.81	47.88
	S	1.61	1.12	2.10	0.64	0.29	0.98	0.18	0.18	0.18	2.39	1.19	3.58	1.61	1.17	2.04	10.62	1.55	19.69
Progesterone	A	0.14	0.12	0.16	0.10	0.10	0.11	0.11	0.09	0.12	0.10	0.10	0.11	0.17	0.11	0.23	0.24	0.18	0.31
	G	0.43	0.29	0.57	0.22	0.17	0.28	0.10	0.10	0.10	0.10	0.10	0.10	0.12	0.09	0.14	0.10	0.10	0.10
	S	0.39	0.18	0.59	0.15	0.12	0.17	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	0.16	0.09	0.23
α -Estradiol	A	0.32	0.02	0.63	0.12	0.08	0.17	0.13	0.00	0.26	0.05	0.03	0.07	0.05	0.03	0.08	0.04	0.03	0.05
	G	74.97	27.61	122.33	5.39	0.015	12.80	161.12	0.015	322.73	0.03	0.02	0.03	0.41	0.30	0.52	0.41	0.31	0.50
	S	3.78	1.19	6.37	0.03	0.02	0.03	0.82	0.03	1.62	0.02	0.01	0.02	0.02	0.02	0.03	0.04	0.03	0.05
α -Testosterone	A	0.06	0.05	0.06	0.18	0.06	0.29	0.05	0.05	0.05	0.85	0.57	1.13	5.99	1.71	10.27	1.14	0.75	1.53
	G	7.81	5.89	9.73	13.54	11.99	15.10	1.99	1.53	2.44	5.35	2.76	7.94	33.31	23.82	42.81	22.51	18.12	26.90
	S	0.26	0.19	0.33	0.58	0.47	0.70	0.26	0.13	0.40	0.07	0.04	0.10	4.78	0.05	9.90	0.10	0.07	0.13
β -Estradiol	A	0.07	0.06	0.08	0.06	0.05	0.06	0.14	0.10	0.19	0.04	0.03	0.05	0.04	0.03	0.04	0.05	0.04	0.06
	G	0.30	0.16	0.43	0.13	0.08	0.18	2.11	0.07	4.25	0.03	0.02	0.05	0.05	0.03	0.06	0.12	0.08	0.15
	S	0.26	0.08	0.43	0.02	0.01	0.02	0.60	0.13	1.06	0.01	0.01	0.01	0.04	0.03	0.05	0.03	0.02	0.05
β -Testosterone	A	0.03	0.03	0.04	0.07	0.06	0.08	0.03	0.03	0.03	0.17	0.09	0.24	0.49	0.03	0.95	0.20	0.16	0.25
	G	0.44	0.19	0.68	3.24	2.63	3.85	0.03	0.03	0.03	0.72	0.34	1.09	3.80	2.30	5.31	4.06	3.15	4.98
	S	0.11	0.06	0.15	0.13	0.10	0.17	0.03	0.03	0.03	0.17	0.11	0.23	1.41	0.49	2.33	0.96	0.39	1.53

Table 4. Misclassification matrix, in the rows the treatment type is given, in the columns the predicted class (in percentage) of the samples after classification

Blank/treatment (class)	Pred 1	Pred 2	Pred 3	Pred 4	Pred 5	Pred 6	No match
Male (1)	86.8	13.2	0	0	0	0	0
Female (2)	7.5	90.8	0.4	0.4	0.9	0	0
DHEA (3)	0	0	100	0	0	0	0
Estradiol (4)	0	0	0	100	0	0	0
Pregnenolone (5)	0	0	0	0	100	0	0
Testosterone (6)	0	0	0	0	0	100	0

Table 5. Overview of the cross-validation of the model by classification of 10 per cent of the data which was omitted from the model.

	Best	Next Best
Female	87.5%	Male
Male	82.6%	Female
DHEA	100%	Male
Estradiol	100%	Female
Pregnenolone	100%	DHEA/Male
Testosterone	100%	DHEA/Male

Table 6. Classification of 20 guaranteed blank samples on the built SIMCA model.

ID	Best
Female, 6-12 months old	Female
Female, 4-6 months pregnant, lactating	Female
Female, 7-9 months pregnant, not lactating	Female
Male, veal calf	Male
Male, mature bull	Male
Female, not pregnant, not lactating	Female
Female, not pregnant, not lactating	Female
Female, 6 months pregnant, lactating	Female
Female, 0-3 months pregnant, lactating	Female
Female, not lactating	Female
Female, pregnant, lactating	Female
Male, young bull	Male
Male, veal calf	Male
Female, heifer, 15-18 months old	Female
Male, fattening bulls *	Pregnenolone
Male, fattening bulls *	Male
Male, fattening bulls *	Male
Male, 24 months olds	Male
Female, 3-4 years old	Female
Female, 6 months old	Female

* Sample consists of a mixture of urine samples from different animals

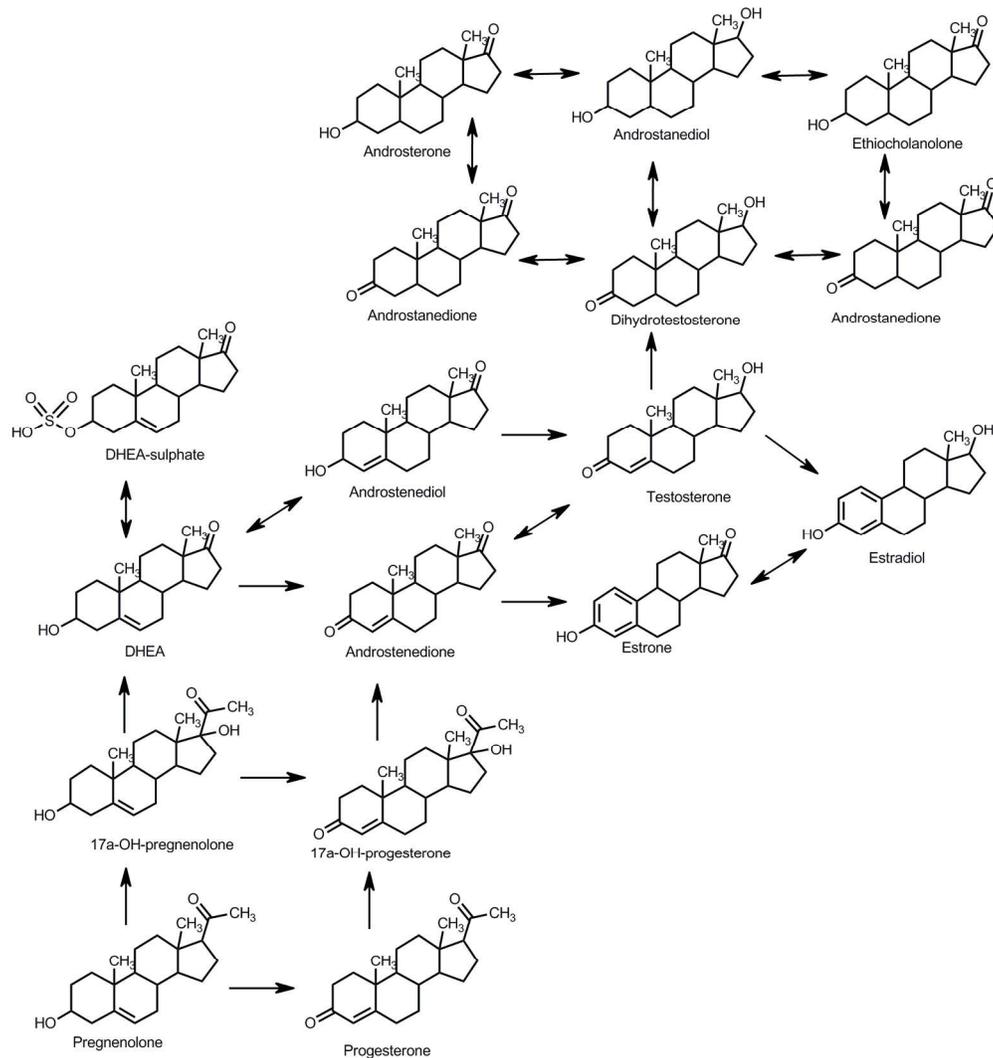


Figure 1. Steroidogenesis of compounds analyzed (steroid aglycons, glucuronide and sulphate conjugates) in this study.

175x187mm (300 x 300 DPI)



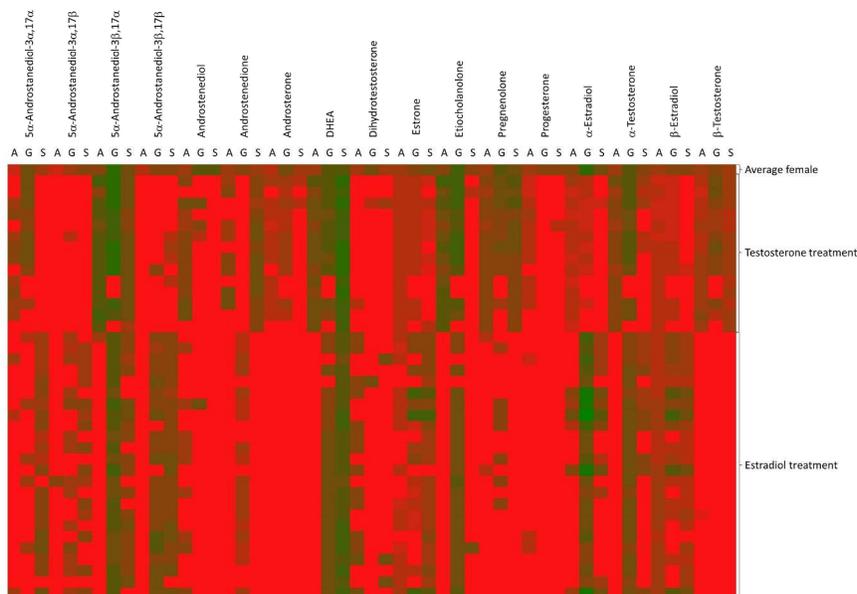


Figure 2. Heatmap of the treatment of female bovine animals with estradiol-benzoate and testosterone-cypionate. Average concentration calculated for 227 female bovine animals. Samples are ranked from the first day of the treatment till the last day of the sampling period after treatment. Dark squares are the highest concentrations and light colors the lowest concentrations. Concentrations were log₁₀ transformed. Color map is equally distributed (16 steps) over all concentrations measured (A=steroid aglycon, G=Glucuronide-conjugate, S=Sulphate-conjugate). 896x597mm (87 x 87 DPI)

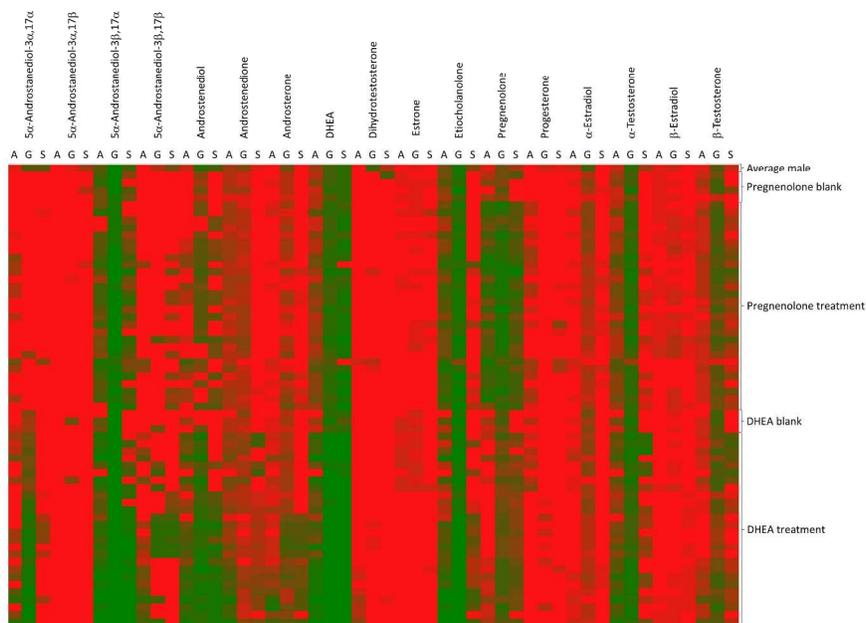


Figure 3. Heatmap of the treatment of six different male bovine animals with DHEA, four different male bovine animals with pregnenolone, samples of the blank control population, and the average concentration for 226 male bovine animals. Dark green squares are the highest concentrations, red squares the lowest. Concentrations were log₁₀ transformed. Color map is equally distributed (16 steps) over all concentrations measured (A=steroid aglycon, G=Glucuronide-conjugate, S=Sulphate-conjugate).
896x597mm (87 x 87 DPI)

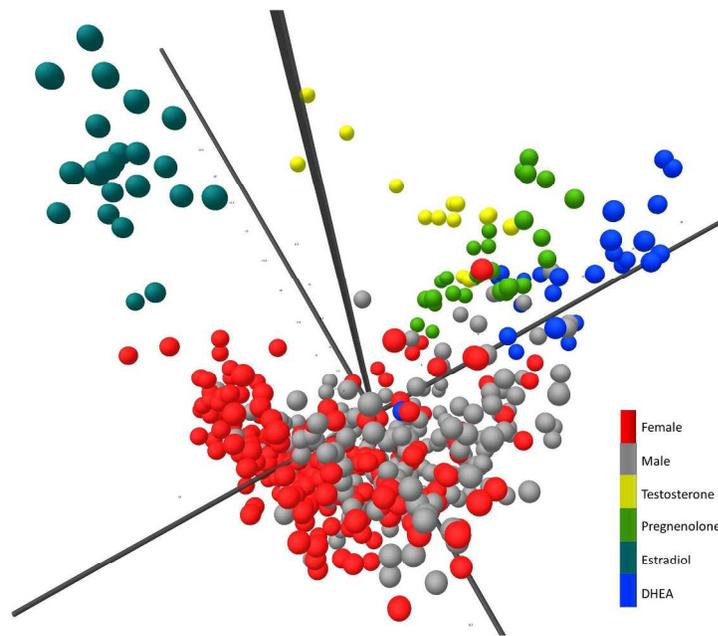


Figure 4. Class projection of the SIMCA analysis of bovine male and female of the control population, estradiol-benzoate, testosterone-cypionate, DHEA and pregnenolone treated animals. See legend for type of sample the colors represent
896x597mm (87 x 87 DPI)