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

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

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Abstract

For years it is suspected that natural hormones are illegally used as growth promoters in cattle in the EU. 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. The 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.

Keywords: natural hormones, abuse, cattle, gas-chromatography tandem mass spectrometry and multivariate statistics
Introduction

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

Natural hormones are metabolized by the liver or intestine (phase I metabolism). Then steroids are converted to more polar compounds by conjugation in order to be excreted via urine from the body (phase II, metabolism). Circulating natural hormone levels are maintained by a dynamic system that ensures homeostasis. In simplest terms, this system can be considered a balancing act between the rate of synthesis and the rate of its metabolic inactivation/elimination. As this system is in balance, both the rate of synthesis and the rate
of metabolic inactivation/elimination have impact on the circulating levels of natural
hormones. Chemically induced disruptions in either of these two processes can potentially
modify circulating natural hormones levels. From a theoretical point of view, administration
of natural hormones will disrupt this balance and will change the excretion patterns.
Therefore, knowledge of the concentrations (Nielen et al. 2007) and the mutual relation of
these compounds in urine is important. The mutual relation is determined in this study via
metabolic profiling. Changes in these patterns can possibly be used as screening tools in
control schemes (Mareck et al. 2008). Metabolic profiling is targeted analysis of metabolites
and intermediates in a (part of a) biological system, typically large datasets are being
acquired. To handle these datasets dedicated statistical tools based on multivariate techniques
are applied, such as Principle Component Analysis (PCA), to elucidate differences between
samples.

The concept of the use of metabolic profiling to detect the illegal use of substances
originates from human sports doping research where the testosterone/epitestosterone ratio
(T/E ratio) (Donike et al. 1983) is applied to demonstrate the abuse of testosterone. An
extension of the T/E ratio in human sport doping research has recently been proposed by
Hemmerbach et al. (1994) and more recently by van Rentherghem et. al (2010a). More ratios
of metabolites from the natural metabolic pathway were included to build a statistical model
to determine dihydrotestosterone (DHT) and (dehydroepiandrosterone) DHEA misuse in
sports. For several metabolites reference concentration values in urine are proposed to
determine if natural steroids are misused (van Rentherghem et al. 2010b).

The feasibility of T/E ratio to determine if synthetic 17ß-testosterone is administrated
to cattle was tested by Angeletti et al., (2006). Due to the higher conversion rate of
testosterone to epitestosterone in cattle this ratio cannot be used. A limited number of
scientific papers is available covering the use of metabolic profiling to detect abuse of natural
hormones in livestock farming. An approach similar to the testosterone/epitestosterone ratio in cattle was introduced by Fritsche et. al. (1998) to distinguish between beef from bulls and beef from steers. They used the masculinity index (MI) which is calculated from testosterone, epitestosterone, and pregnenolone concentrations in meat samples. By means of the MI, a classification of a beef sample of unknown origin was performed, regardless of the slaughter age of the animal. However, the MI cannot be used to indicate abuse of natural hormones. Le Bizec et. al (2006) described the measurement of natural occurring steroids in kidney samples before and after treatment with natural hormones. By applying discriminant statistics they were able to separate the animals treated with natural hormones from the non-treated animals. A similar approach was developed by Scarth et. al.(2010; 2011), by measuring concentrations of natural hormones and markers in urine from cattle that was not treated with natural hormones. As indicated in the reviews of Scarth et. al. (Scarth et al. 2009) and Mooney et. al. (2008) important topics in the field of steroid abuse are e.g. validation of the methods and introduction of high throughput analysis. Furthermore they suggested that more research should be conducted on the applicability of multivariate statistical approaches to detect steroid abuse in cattle.

Aim of the present study was to develop a urine analysis method that is capable of discriminating between animals treated with natural hormones and untreated animals. The sample clean-up method developed in this study was based on previously (Tricht et al. 2008) developed method which analyzes natural hormones in serum. Detection of the individual compounds is based on gas-chromatography tandem mass spectrometry (GC-MS/MS), covering the steroidogenesis of almost all major natural (pro)hormones, as depicted in Figure 1.
A large number of urine samples – collected at farms in the Netherlands and from cattle treated with natural hormones – were analyzed using this method. The data collected were used to build a statistical model that can identify possible abuse of natural hormones.

**Materials and methods**

**Bovine urine samples collected at farms**

Bovine urine samples were obtained from Dutch routine control programs in the period of 2008 and 2009. Information on the animals such as age and gender was registered, but the origin of the samples was kept anonymous. Since the samples were collected at farms all over the Netherlands it cannot be guaranteed that all were from non-treated animals. Since the population is large and the average rate of animals testing positive in EU monitoring programs is low the assumption that most of the samples are from animals which were not treated with any growth promotors is justified. After sample collection the urine samples were stored at -20°C until analysis. In total 620 urine samples were collected in order to obtain a sufficiently large population to perform a reliable statistical evaluation. From the 620 samples 510 were used to build the assumed untreated reference collection, the others 110 were divided over the different animal experiments; estradiol-benzoate n=35 testosterone-cypionate n=14, DHEA n=29, pregnenolone n=32.

**Controlled animal treatment experiments**

Urine samples from different animal studies were used. The studies were performed following ethical approval at either CVI, Lelystad, the Netherlands or University of Ghent, Belgium.
Estradiol-benzoate treatment

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

Testosterone-cypionate treatment

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

DHEA-treatment

In a time span of one and a half year, three independent bovine DHEA treatment experiments were performed using identical treatment and sampling schedules. Male Friesian bovines were purchased at the local market and housed for 2–3 weeks before the start of each experiment. Each of the three experiments consisted of two animals of which one was treated orally by capsules containing 1000 mg of DHEA, and the other intramuscular by 1000 mg of
DHEA dissolved in 10 mL of Miglyol 812. Repeated dose administrations were performed seven times at 24h intervals. Before the start of the treatment urine collections were made, and during the animal trials urine was sampled at days 2, 5, and 7. Untreated control animals were included in all three experiments; three animals in the first experiment, one in the second and two in the third experiment.

**Pregnenolone-treatment**

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

**Determination of specific density and total solid content**

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

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

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

**Materials**

Standard solutions of 1 µg L⁻¹ the analytes (table 1) were prepared in ethanol. Thousand- and
ten thousand-fold dilutions of the standards were made and stored at -20°C until analysis. Methanol and ethanol were obtained from (Biosolve, the Netherlands). Acetone, acetonitrile, ethanol, n-pentane, iso-octane, dry ethyl acetate and Tris(hydroxymethyl)-amino-methane were of analytical grade and obtained from Merck (MO, USA). N-Methyl-N-trimethylsilyl-trifluor(o)acetamide (MSTFA) was obtained from Alltech (IL, USA), ammonium iodide was obtained from Fluka (MO, USA), dithiothreitol was obtained from AnalaR (IL, USA). β-Glucuronidase was obtained from Roche diagnostics (Switzerland). Derivatization reagent (MSTFA++) consisted of N-methyl-N-trimethylsilyl trifluoroacetamide/ammonium iodide/dithiothreitol (1000:2:4, v/w/w). Demineralized water was obtained from a milli-Q purification system. Bond Elut C18 500 mg (3ml) SPE columns were obtained from Varian (CA, USA). Set of guaranteed blank bovine reference urines, bov01 to bov20 (Sterk, S et al. 1998), was obtained from EURL for residues (the Netherlands).

**GC-MS/MS analysis**

A Varian 1200L triple quadrupole mass spectrometer equipped with a CP8400 autosampler and a CP-3800 GC was used. The GC column was a VF-17MS (L = 30 m, id = 0.25 mm, df = 0.25 µm), obtained from Varian. Two microliter of the purified samples or standard solutions was splitless injected onto the GC column at a pulsed pressure of 30 psi. The temperature program started at 110°C (constant for 1 min), increased 20°C.min-1 to 240°C and was held for 1.5 min. Then, the temperature was increased 1°C.min-1 to 244°C. Finally, the temperature was increased 25°C.min-1 to 340°C. This temperature was held for 2 min. The helium flow was kept constant at 1.0 ml.min-1. The GC-MS/MS was operated in electron ionization (EI) mode using Multiple Reaction Monitoring (MRM). In Table 1 the ion transitions monitored and the applied collision energy for each ion transitions is shown for all compounds analyzed.
**Analytical procedure**

**Solid phase and liquid-liquid extraction**

To 2 ml of urine 1 ml of water was added and next 5 ng of internal standard mixture by adding 50 µl of 0.1 ng ul-1 internal standard mixture. The samples were loaded onto a preconditioned (3 ml of methanol and 3 ml of milli-Q water) C\textsubscript{18} disposable solid phase extraction (SPE) column. The SPE C\textsubscript{18} column was washed with 3 ml of milli-Q water. The conjugated compounds were eluted with 3 ml 35/65 (%-v/v) acetonitrile/water; this eluate was denoted glucuronide and sulphate fraction (G+S) and further processed as described in the paragraph deconjugation and isolation of glucuronide conjugates. Next, the unconjugated steroids (steroid aglycons) were eluted with 3 ml of acetone; that fraction contained steroid steroid aglycons and was denoted A. Eluate A was evaporated at 50°C under a gentle stream of nitrogen until nearly dry and re-dissolved in 100 µl of methanol and 2 ml of TRIS-buffer at pH 9.2. Liquid Liquid Extraction (LLE) was performed twice with 7 ml of n-pentane. The mixture was centrifuged for 5 minutes at 3000 g. The organic layer was collected in a glass tube and evaporated to dryness at 55°C under a gentle stream of nitrogen and further processed as described in the paragraph derivatization.

**Deconjugation and isolation of glucuronide conjugates**

The G + S fraction was dried at 55°C under a gentle stream of nitrogen and dissolved in 1 mL of phosphate buffer (pH 7.4). 5 ng of internal standard mixture (see table 1) and 10 µL of β-glucuronidase was added. The mixture was vortexed and hydrolyzed for three hours at 55°C. An SPE C\textsubscript{18} column (3 mL) was preconditioned with 3 mL of methanol followed by 3 mL of water. After that, 2 ml water was added to the SPE column and the hydrolyzed sample was applied. The column was washed with 3 mL water. The sulphate-conjugates were eluted with 3 mL of 35:65 (v/v, %) acetonitrile:water. The eluate collected, which was denoted fraction S,
was evaporated to dryness at 55°C under a gentle stream of nitrogen and further processed as described in paragraph deconjugation of sulphate conjugates. The deconjugated compounds were eluted with 3 mL of acetone; this fraction was denoted G. The eluate was evaporated to dryness at 55°C under a gentle stream of nitrogen and further processed as described in the paragraph derivatization.

Deconjugation of sulphate conjugates

To the dried extract (fraction S) 5 ng of internal standard mixture and 1 mL of dry ethyl acetate and 2 µL 4M H$_2$SO$_4$ were added. The solvolysis was performed for 1 hour at 40 °C after which 4 mL of ethyl acetate and 1 mL of an aqueous 5% NaHCO$_3$ solution were added. The mixture was vortexed and centrifuged. The water phase was removed. The ethyl acetate layer was transferred to a clean glass tube and evaporated to dryness at 55°C under a gentle stream of nitrogen. The residue was dissolved in 100 µl of methanol and 2 mL of TRIS buffer pH 9.2 was added. LLE was performed twice with 7 ml n-pentane. The pentane fraction was evaporated to dryness at 55 °C under a gentle stream of nitrogen and further processed as described in the paragraph derivatization.

Derivatization

The dried residues (fraction A, G and S) were derivatized separately. First they were dissolved in 0.5 ml of ethanol and transferred into a derivatization-vial and evaporated to dryness at 50°C under a gentle stream of nitrogen. The dry residue was derivatized by adding 25 µl of MSTFA++ followed by incubation of 1 hour at 60°C. The derivatized mixture was evaporated to dryness at 50°C under nitrogen. The dry residue was reconstituted in 25 µl of iso-octane.
Validation of the method

Validation was performed for all compounds in Table 1. For these compounds the CC$_{\alpha}$, CC$_{\beta}$ and measurement uncertainty were determined. The decision limit (CC$_{\alpha}$) and detection capability (CC$_{\beta}$) (2002/657/EC) were determined using a three point standard calibration curve in different blank urine samples. Each individual sample was analyzed six-fold on each day. The measurement was repeated on three different days. From this calibration curve the y-intercept and slope were calculated ($y=ax+b$). CC$_{\alpha}$ is the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the y-intercept ($\alpha=1\%$). The calculated CC$_{\alpha}$ was checked by spiking samples at the corresponding levels. The CC$_{\beta}$ is the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the y-intercept (B=5%). The (within day) repeatability and within laboratory reproducibility (between days) were determined from this dataset using the approach as described in ISO 5725 (ISO 5725, 1994). To determine the measurement uncertainty all variances were summed. For this validation the variances are equal to the within days reproducibility. The measurement uncertainty at the different spiking levels is calculated with the equation: measurement uncertainty $U = 2S_R$, with $S_R$ being the sum of the variances. All the variances are expressed as a coefficient of variance.

Data Processing

GC-MS/MS Data was automatically processed using MS Workstation$^\circledR$ software from Varian. All peak integrations were manually checked and baseline corrected if necessary. Concentrations below the CC$_{\alpha}$ were rejected and not used for the statistical evaluation of the
results. If the measured concentration was outside the calibration range the sample was re-
analyzed following dilution.

Statistical Analysis of the data

Multivariate analyses were performed using Pirouette version 4.0 from Infometrix® (Bothell, Washington, USA) The data was first explored using Principle Component Analysis (PCA), showing separation between all groups used in this study, e.g. blank bovine female and male, testosterone, estradiol, DHEA and pregnenolone treatment. However, PCA is an
unsupervised model and better suited supervised statistical models are available to perform
pattern recognition. A start was made with a number of objects whose group membership is
known, for example testosterone treated animals. These objects were considered the
‘learning’ or ‘training’ objects (Miller, J et al. 2005). The aim of supervised pattern
recognition methods is to use these objects to find a rule for allocating new objects of the
unknown group to the correct group. A number of supervised statistical models were
explored. K-nearest neighbor method, (linear) discriminant analysis and SIMCA. All data
was mean-centered and log_{10} transformed to suppress exorbitant values.

Results and Discussion

Validation of the method

The described method was validated as a quantitative confirmatory method according to
Commission Decision 2002/657/EC (2002/657/EC, CD 2002). The analyses were performed
in the same way as routine analyses of unknown samples, with the addition of internal
standards. Because sulphate and glucuronide standards are in contrast with steroid steroid
aglycons hardly commercially available it is not possible to fully validate the method for
these conjugates. As samples are generally cleaner after the first SPE step, it is expected that
the CCα and CCβ are in the same range as they would be for the steroid steroid aglycons. Therefore the same CCα and CCβ are used in this study for the steroid steroid aglycons and conjugated compounds. Several performance characteristics were determined. Important parameters are the decision limit (CCα), the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant, and the detection capability (CCβ), the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – β. In Table 2 an overview is given of the validation results.

The results of the validation confirm that GC-MS/MS measurement of a large group of (pro)hormones is feasible. For all natural steroids measured the analytical parameters are typical for methods used for residue analysis in bovine urine with CCα for all compounds in the range of 0.007-0.46 ng.ml⁻¹. The measurement of uncertainty is for most compounds relative high. There are two main reasons for this, all measured compounds occur endogenous in samples of urine which of course attributes to the variation, and it can be expected that the variability is relative high because the developed method is capable to measure steroids at very low pg.ml⁻¹ levels. It is difficult to compare the validation results with two recent studies (Regal et al. 2009; Scarth et al. 2010) of comparable goal and methods. These studies different by matrix, serum instead of urine, and the validation was not performed according to Commission Decision 2002/657(2002).
Quantitative urine analysis

In total, almost 620 samples were analyzed for the compounds given in Table 2. For all compounds the concentrations of the steroid aglycon, glucuronide and sulphate fractions were determined. See for the average values and the 95% Confidence Interval (CI) for the male and female population, and for the treated animals table 3.

In theory, treatment with natural hormones should change the mutual relations and therefore influence the typical concentrations of steroids involved in the steroidogenesis. To visualize the effects of administration of natural hormones on their concentrations in urine and to explore the data as depicted in table 3, heatmap plots (figure 2 and 3) were constructed. Treatment of female and male animals with different natural steroids and the average concentration values of all male and female animals from the reference population are depicted in figures 2 and 3.

Testosterone treatment (female bovine)

The heatmap in figure 2 gives a quick overview of the increase in concentrations of different natural hormones after treatment. The concentrations of the steroid aglycon, glucuronide and sulphate of 17ß-testosterone and its major metabolite 17α-testosterone are increased and slowly decrease over time. Possibly because the levels of testosterone-cypionate in the injection depots decreases. Most of the testosterone is rapidly metabolized (figure 1) to the
steroid aglycon and glucuronide of ethiocholanolone; the steroid aglycon, glucuronide and
sulphate of 5α-androstenediol-3β,17α; and androstenedione-sulphate. These effects can be
expected since this is the metabolism pathway of testosterone. When the heatmap is further
examined some other effects are observed. There is an increase in the levels of all three forms
of DHEA and especially the steroid aglycon form of DHEA. This is not expected since
DHEA is a precursor of testosterone and not a metabolite. Furthermore there is an increase in
the steroid aglycon form of androstenediol and pregnenolone. This suggests some sort of
feedback mechanism, which to the best of our knowledge, was not observed in other studies.
A possible explanation is that the biosynthesis from androstenediol to testosterone is inhibited
due to the high exogenous level of testosterone, inducing an overproduction of all steroids
involved in this pathway (see Figure 1), immediately following injection of testosterone-
cypionate. This would also increase the production of the hormone DHEA-sulphate. We do
not know if this effect is limited to the particular animal used in this study or that these
effects are generic in female bovine animals treated with testosterone-cypionate. To
determine the excretion and feedback routes of testosterone administration on DHEA and
other steroids more experiments are needed. The following experiments could be performed;
labeling (2H, 13C) of administrated steroids, the use of IRMS, however, low concentrations
will limit the use of IRMS, or the use of 14C labeling and analysis by Accelerator Mass
Spectrometry (AMS).

**Estradiol treatment (female bovine)**

Treatment of a female bovine animal with 17β-estradiol-3-benzoate showed less pronounced
effects when the heat-map (figure 2) is examined. There is an increase in the concentrations
of the steroid aglycon, glucuronide and sulphate of 17β-estradiol and its major metabolite
17α-estradiol. Estradiol itself is metabolized to estrone (figure 1). Therefore, an increase in
the concentration of estrone-glucuronide is observed. The levels of estradiol and estrone
decrease over time possibly because the concentration of estradiol-benzoate in the injection
depot decreases over time. Less pronounced is the increased production of testosterone-
sulphate, 5α-androstenediol-3β,17α-glucuronide and 5α-androstenediol-3α,17α-sulphate
indicating possible feedback of testosterone via this route because the route to estradiol is
inhibited due to the increased concentration of exogenous 17α-estradiol. Since these results
are from the treatment of only one animal no general conclusions can be made on the
metabolism pattern for estradiol-benzoate treatment. However, this treatment again
demonstrates that besides the concentrations changes of other compounds, the administered
natural hormone and its metabolites change as well, in accordance with expectations.

(figure 3)

DHEA treatment (male bovine)

After treatment with DHEA there is an increase in the concentrations of the steroid aglycon,
androstenediol, α-testosterone and 5α-androstenediol-3β,17α. There also is a slight increase in concentration of
some other metabolites, e.g: androsterone-sulphate, ethiocholanolone-glucuronide and 5α-
androstanediol-3α,17α-glucuronide. All these steroids are metabolites from DHEA and
clearly demonstrate the clearance of DHEA after treatment. Some of the detected metabolites
were previously suggested as DHEA metabolites in an LC-ToF-MS study (Rijk et al. 2009).

Less pronounced, but also observed in this treatment is the feedback towards
precursors of DHEA: there is a slight increase in concentration of pregnenolone-glucuronide
and pregnenolone-sulphate. These effects are observed in all animals included in this study,
some animals react stronger than others on the treatment; this difference is probably caused
by the type of treatment, intramuscular or oral, and/or on the metabolism rate of the particular
animal.

**Pregnenolone treatment (male bovine)**

The effect of treatment of the male bovine animals with pregnenolone is less pronounced than
other treatments studied. An increase in the concentrations of the steroid aglycon,
gluconide and sulphate of pregnenolone is observed, and there also seems to be a minor
effect on the steroid aglycons of progesterone and DHEA. This increase in concentration of
these steroid aglycons can be explained by the metabolic route of pregnenolone. Although the
changes in hormone concentrations are less clear than for the previously described
treatments, subtle changes are observed in the metabolism pattern after treatment with
pregnenolone.

**Multivariate statistics of the data**

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

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

From the class projection of the SIMCA analysis (fig. 4), several observations can be made.
There are differences between the groups, especially between the treated and non-treated
animals. All groups are separated to a certain extent requiring the first three principle
components. This separation indicates that the model can be used to predict which group
unknown samples belong to. Since the groups are not perfectly separated, the classification
will most likely not be perfect for all samples. In particular for the separation of the male and
female untreated groups. The predictive power of the model is described by the
misclassification (or confusion) matrix. The misclassification matrix describes how the data
used to build the model would fit on the model. Applying the constructed SIMCA model
makes it possible to determine to which group a certain animal belongs; male, female or
treated with compound X. A unique feature of SIMCA is that samples can also be classified
as belonging to none of the groups, indicating an unknown metabolic pattern or treatment. If
a sample is classified close to another group during classification it will be indicated as “next
best”. This next best score has to be taken into account when samples are classified. A
perfectly classified sample has for the next best score zero. The misclassification matrix of
the data set is shown in table 4.

From table 4 it can be concluded that the prediction for treated animals is excellent. The
scores of the male and female control group are not 100% correct. This can be explained by
the fact that the female and male control group are less homogenous compared to the animals in the treated groups. Also are a few female samples classified in a treated group. This can be caused by natural variations in steroid profiles which causes a sample to be incorrectly classified or by the fact that we cannot be sure that all samples of the reference population used were untreated. The steroid profiles of the male and female groups are influenced by age, animal breed, and external influences such as e.g. food and housing. If the reference population would be smaller and more homogenous, the prediction values would have definitely been better. However, the ultimate goal is to use the model for control programs. Therefore it is necessary to include more different treated and non-treated bovine animals in the model. It is not a major problem if the gender of an animal is not correctly assessed; it is far more important that treated animals differentiate from a normal population of animals. With this model it is possible to classify treated animals correctly. There is a chance that the number of misclassifications in the treated animal population increases if more different treatments are included in the model. On the other hand, more samples from treated animals make it possible to further refine the model and therefore increase its predictive power.

**Cross-validation of the model**

The model was validated by means of cross-validation. Cross-validation is a variant of classification analysis where, particular cases are removed from the data set, the reduced data set is then used to classify the omitted cases as if it were new cases. For validation of the model, 10% of the samples were removed from the model. The model was then built as described before with 90% of the data and 10% removed samples were classified. In table 5 an overview is given of this validation.

(table 5)
With the 10% holdout all samples from the treated animals could be successful classified correctly using the SIMCA model. The next best classification for DHEA and estradiol is corresponding to the correct gender. The next best classification for both pregnenolone and testosterone is DHEA or male. In the heat-map of the pregnenolone treatment (fig. 3) can be seen that there is some increase in the steroid aglycon of DHEA and testosterone which could explain the classification in the male (higher testosterone levels) and DHEA group. The next best classification for testosterone is DHEA or male. This is less surprising, from the heatmap [fig. 3] it can be observed that there is an increase in the steroid aglycon of DHEA resulting in classification in the DHEA group. An increase in testosterone after treatment with this compound is shown resulting in classification in the male group. Classification of the male and female animals in the opposite sex group can be expected from the results of the misclassification matrix. An explanation can be the age of the animals. Young animals have very low concentration in sex steroids.

To further evaluate the model, twenty guaranteed blank bovine samples were analyzed and classified. The blank bovine sample set consists of different breeds, genders and ages. Using this set, the model was verified for its accuracy towards classification of samples which were not included while building the model. It is also important for control purposes to establish that the model has a low false positive score. The results of the validation with the blank sample set are shown in table 6.

The results of the classification of guaranteed blank bovine samples are satisfying for this type of model. Only one sample was not assigned to the correct group; in this case a mixture of bovine samples which is assigned to the pregnenolone group. Probably this is due the fact that mixed urine was used, resulting in a profile that correspond with a treated
animal. All other samples were assigned correctly which indicates that the number of false positive samples in screening is low when the model is used in a routine control programs.

**General discussion**

In a recent study (Anizan et al. 2011a) the glucuronide and sulphate conjugate concentrations were used to predict natural hormone abuse. In this study (Anizan et al. 2011a) the ratio of

\[
\text{[sum of (Epiandrosterone-Glucuronide + Androsterone-Sulphate + Epiandrosterone-Sulphate + alpha-Testosterone-Glucuronide + Etiocholanolone-Glucuronide) over DHEA-Sulphate]}
\]

was calculated to determine whether treatment with the natural hormones androstenedione had occurred. In another study (Anizan et al. 2011b) glucuronide and sulphate metabolites were detected using precursor ion scan acquisition mode. This approach was tested using urine from animals treated with 4-androstenedione. A statistical model was build that could be used to detect abuse of 4-androstenedione. The basic idea, to measure the concentration of glucuronides and sulphates steroids, is comparable to our study, however, its implementation is different. The concentrations of steroid aglycons were in both studies (Anizan et al. 2011a; Anizan et al. 2011b) not taken into account. It is important to monitor steroid aglycon concentrations because their concentrations will also increase in case an animal is treated with a natural hormone. The described methods furthermore lack performance characteristics.

The use of ratios of steroids and prohormones to determine abuse seems promising; in human doping studies the use of ratios has been extensively examined by van Renthergem (2010a). For a large population the concentrations of steroids and precursors were determined. Combinations of ratios were statistically compared to determine if abuse of natural hormones had occurred. It was concluded that the use of ratios of several metabolites could help the decision making to determine if abuse with natural hormones had occurred. However, the use of ratios was found to be limited in cattle due to large individual variations
(Angeletti et al., 2006). Another approach is the use of statistical multivariate analysis to determine abuse of natural hormones as was demonstrated by Regal (Regal et al. 2009). In this study a multivariate model based on orthogonal projections to latent structures (OPLS) was built, based on high resolution accurate mass spectra LC runs of treated and non-treated animals. The model was used to determine what type of application form of estradiol was used. The OPLS model was built on non-identified markers. This approach was used successful in this study but has to be further evaluated by adding more non-treated animals from control programs. However, this study demonstrates that statistical models can be predictive for the type of hormone treatment.

Conclusion

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

Treatment of bovine animals with natural steroids results in increased concentrations of the steroid aglycon of administered compounds, as well as their glucuronide- and sulphate conjugates. Furthermore, an increase in the concentration is observed for the compounds involved in the major metabolic pathways of the administered compounds. In some cases there is an increase in the glucuronide or sulphate conjugates of a metabolite related to the excretion of the compounds. It was observed that there is a feedback mechanism resulting in increasing concentrations of some of the precursors of the administered compounds. This was rather surprising and to our knowledge not observed before. The obtained results were used
to build a model based on SIMCA. The model was validated by means of a cross-validation
and by analyzing a set of guaranteed blank urine samples of bovine animals. Validation
indicated that the model can be used to classify animals in a treated and untreated group. We
can conclude that the statistical model is a promising strategy to determine whether bovine
animals are treated with natural hormones. This model can be used as a screening method to
pinpoint suspect samples. Samples that are regarded as suspect, should be confirmed with,
preferably, isotope ratio mass spectrometry or with other additional research.

More data from different animals treated with natural hormones must be added to the
database to make the model more robust. For use in routine monitoring programs, the model
needs simplifications, e.g. by omitting non-discriminative compounds from the GC-MS/MS
quantitative analysis. Possibly the model can be used to investigate treatments with synthetic
exogenous growth promotors such as stanozolol, since these also will influence the
steroidogenesis and are known for feedback effects.

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DHEA misuse in sports." Drug testing and analysis 2(11-12): 582.
Figure 1. Steroidogenesis of compounds analyzed (steroid aglycons, glucuronide and sulphate conjugates) in this study.

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 log_{10} transformed. Color map is equally distributed (16 steps) over all concentrations measured (A=steroid aglycon, G=Glucuronide-conjugate, S=Sulphate-conjugate).

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).

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

<table>
<thead>
<tr>
<th>ID</th>
<th>Source</th>
<th>MRM1</th>
<th>CE (V)</th>
<th>MRM2</th>
<th>CE (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>Searle</td>
<td>460 &gt; 445</td>
<td>-8.0</td>
<td>460 &gt; 157</td>
<td>-25.0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Steraloids</td>
<td>458 &gt; 443</td>
<td>-7.5</td>
<td>458 &gt; 157</td>
<td>-20.0</td>
</tr>
<tr>
<td>Progesterone-13C2</td>
<td>EURL</td>
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<td>-8.0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>DHEA</td>
<td>Steraloids</td>
<td>432 &gt; 417</td>
<td>-7.5</td>
<td>432 &gt; 327</td>
<td>-15.0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>Organon</td>
<td>430 &gt; 415</td>
<td>-10.0</td>
<td>430 &gt; 209</td>
<td>-12.5</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>Sigma</td>
<td>434 &gt; 344</td>
<td>-5.0</td>
<td>434 &gt; 239</td>
<td>-25.0</td>
</tr>
<tr>
<td>17β-Testosterone</td>
<td>Steraloids</td>
<td>432 &gt; 301</td>
<td>-15.0</td>
<td>432 &gt; 209</td>
<td>-12.5</td>
</tr>
<tr>
<td>17α-Testosterone</td>
<td>Steraloids</td>
<td>432 &gt; 327</td>
<td>-15.0</td>
<td>432 &gt; 209</td>
<td>-12.5</td>
</tr>
<tr>
<td>17β-Testosterone-d3</td>
<td>EURL</td>
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<td>-13.0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dihydrotestosterone</td>
<td>Sigma</td>
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<td>-7.5</td>
<td>434 &gt; 195</td>
<td>-22.5</td>
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<td>Steraloids</td>
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<td>414 &gt; 155</td>
<td>-17.5</td>
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<td>17β-Estradiol</td>
<td>Diosynth</td>
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<td>416 &gt; 285</td>
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<td>-7.5</td>
<td>416 &gt; 285</td>
<td>-10.0</td>
</tr>
<tr>
<td>17β-Estradiol-d3</td>
<td>EURL</td>
<td>419 &gt; 285</td>
<td>-10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Etiocholanolone</td>
<td>Sigma</td>
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<td>-10.0</td>
<td>434 &gt; 329</td>
<td>-20.0</td>
</tr>
<tr>
<td>Androsterone</td>
<td>EURL</td>
<td>434 &gt; 419</td>
<td>-10.0</td>
<td>434 &gt; 329</td>
<td>-20.0</td>
</tr>
<tr>
<td>5α-Androstanediol-3β-17β</td>
<td>Steraloids</td>
<td>436 &gt; 241</td>
<td>-15.0</td>
<td>436 &gt; 346</td>
<td>-5.0</td>
</tr>
<tr>
<td>5α-Androstanediol-3β-17α</td>
<td>Steraloids</td>
<td>436 &gt; 241</td>
<td>-15.0</td>
<td>436 &gt; 346</td>
<td>-5.0</td>
</tr>
<tr>
<td>5α-Androstanediol-3α-17β</td>
<td>Steraloids</td>
<td>436 &gt; 241</td>
<td>-15.0</td>
<td>436 &gt; 346</td>
<td>-5.0</td>
</tr>
<tr>
<td>5α-Androstanediol-3α-17α</td>
<td>Steraloids</td>
<td>436 &gt; 241</td>
<td>-15.0</td>
<td>436 &gt; 346</td>
<td>-5.0</td>
</tr>
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</table>
Table 2. Overview of the validation results for the determination of steroids in bovine urine by GC-MS/MS, \( U = \) measurement of uncertainty (\( k=2 \))

<table>
<thead>
<tr>
<th>ID</th>
<th>CC( \alpha ) (ng.ml(^{-1} ))</th>
<th>CC( \beta ) (ng.ml(^{-1} ))</th>
<th>U (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnenolone</td>
<td>0.18</td>
<td>0.30</td>
<td>68.7</td>
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<td>Progesterone</td>
<td>0.10</td>
<td>0.17</td>
<td>24.1</td>
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<td>DHEA</td>
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<td>0.10</td>
<td>39.4</td>
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<td>0.20</td>
<td>88.0</td>
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<td>0.04</td>
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<tr>
<td>17α-Testosterone</td>
<td>0.05</td>
<td>0.08</td>
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<td>0.10</td>
<td>0.18</td>
<td>71.3</td>
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<td>Estrone</td>
<td>0.10</td>
<td>0.17</td>
<td>109</td>
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<td>17β-Estradiol</td>
<td>0.007</td>
<td>0.01</td>
<td>11.4</td>
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<td>17α-Estradiol</td>
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<td>0.02</td>
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<td>0.10</td>
<td>46.0</td>
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<td>Androsterone</td>
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<td>0.11</td>
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<td>5α-Androstanediol-3β, 17β</td>
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<td>0.34</td>
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<td>0.45</td>
<td>57.8</td>
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</table>
Table 3. Average concentration (µg.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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conjugate</th>
<th>Female</th>
<th>Male</th>
<th>B-Estradiol-17ß</th>
<th>B-Testosterone-cypionate</th>
<th>DHEA</th>
<th>Pregnenolone</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>±SD</td>
<td>CV (%)</td>
<td>Average</td>
<td>±SD</td>
<td>CV (%)</td>
</tr>
<tr>
<td>5α-Androstanediol-3α,17β</td>
<td>A</td>
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<td>±0.26</td>
<td>100</td>
<td>0.26</td>
<td>±0.54</td>
<td>200</td>
</tr>
<tr>
<td>G</td>
<td>0.26</td>
<td>±0.26</td>
<td>100</td>
<td>0.26</td>
<td>±0.54</td>
<td>200</td>
<td>0.31</td>
</tr>
<tr>
<td>S</td>
<td>0.26</td>
<td>±0.26</td>
<td>100</td>
<td>0.26</td>
<td>±0.54</td>
<td>200</td>
<td>0.31</td>
</tr>
<tr>
<td>5α-Androstanediol-3β,17β</td>
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<td>0.46</td>
<td>±0.46</td>
<td>100</td>
<td>0.46</td>
<td>±0.46</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>0.46</td>
<td>±0.46</td>
<td>100</td>
<td>0.46</td>
<td>±0.46</td>
<td>100</td>
<td>0.46</td>
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<tr>
<td>S</td>
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<td>±0.46</td>
<td>100</td>
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<td>±0.03</td>
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<td>±0.32</td>
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<td>G</td>
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<td>±0.03</td>
<td>100</td>
<td>0.18</td>
<td>±0.32</td>
<td>100</td>
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<tr>
<td>S</td>
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<td>±0.03</td>
<td>100</td>
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<td>±0.47</td>
<td>100</td>
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<tr>
<td>G</td>
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<td>±0.47</td>
<td>100</td>
<td>0.47</td>
<td>±0.47</td>
<td>100</td>
<td>0.47</td>
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<tr>
<td>S</td>
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<td>±0.47</td>
<td>100</td>
<td>0.47</td>
<td>±0.47</td>
<td>100</td>
<td>0.47</td>
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<tr>
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<td>±0.06</td>
<td>100</td>
<td>0.05</td>
<td>±0.05</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
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<td>±0.06</td>
<td>100</td>
<td>0.05</td>
<td>±0.05</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>S</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.05</td>
<td>±0.05</td>
<td>100</td>
<td>0.05</td>
</tr>
<tr>
<td>DHEA</td>
<td>A</td>
<td>0.10</td>
<td>±0.10</td>
<td>100</td>
<td>0.12</td>
<td>±0.12</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>0.10</td>
<td>±0.10</td>
<td>100</td>
<td>0.12</td>
<td>±0.12</td>
<td>100</td>
<td>0.13</td>
</tr>
<tr>
<td>S</td>
<td>0.10</td>
<td>±0.10</td>
<td>100</td>
<td>0.12</td>
<td>±0.12</td>
<td>100</td>
<td>0.13</td>
</tr>
<tr>
<td>Estrone</td>
<td>A</td>
<td>0.32</td>
<td>±0.32</td>
<td>100</td>
<td>0.42</td>
<td>±0.42</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>0.32</td>
<td>±0.32</td>
<td>100</td>
<td>0.42</td>
<td>±0.42</td>
<td>100</td>
<td>0.51</td>
</tr>
<tr>
<td>S</td>
<td>0.32</td>
<td>±0.32</td>
<td>100</td>
<td>0.42</td>
<td>±0.42</td>
<td>100</td>
<td>0.51</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>A</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>S</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
<td>0.08</td>
<td>±0.08</td>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>Testosterone</td>
<td>A</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
</tr>
<tr>
<td>G</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.06</td>
</tr>
<tr>
<td>S</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.06</td>
<td>±0.06</td>
<td>100</td>
<td>0.06</td>
</tr>
</tbody>
</table>
**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

<table>
<thead>
<tr>
<th>Blank/treatment (class)</th>
<th>Pred 1</th>
<th>Pred 2</th>
<th>Pred 3</th>
<th>Pred 4</th>
<th>Pred 5</th>
<th>Pred 6</th>
<th>No match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (1)</td>
<td>86.8</td>
<td>13.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Female (2)</td>
<td>7.5</td>
<td>90.8</td>
<td>0.4</td>
<td>0.4</td>
<td>0.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DHEA (3)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Estradiol (4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pregnenolone (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Testosterone (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th></th>
<th>Best</th>
<th>Next Best</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>87.5%</td>
<td>Male</td>
</tr>
<tr>
<td>Male</td>
<td>82.6%</td>
<td>Female</td>
</tr>
<tr>
<td>DHEA</td>
<td>100%</td>
<td>Male</td>
</tr>
<tr>
<td>Estradiol</td>
<td>100%</td>
<td>Female</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>100%</td>
<td>DHEA/Male</td>
</tr>
<tr>
<td>Testosterone</td>
<td>100%</td>
<td>DHEA/Male</td>
</tr>
</tbody>
</table>
Table 6. Classification of 20 guaranteed blank samples on the built SIMCA model.

<table>
<thead>
<tr>
<th>ID</th>
<th>Best</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, 6-12 months old</td>
<td>Female</td>
</tr>
<tr>
<td>Female, 4-6 months pregnant, lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, 7-9 months pregnant, not lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Male, veal calf</td>
<td>Male</td>
</tr>
<tr>
<td>Male, mature bull</td>
<td>Male</td>
</tr>
<tr>
<td>Female, not pregnant, not lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, not pregnant, not lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, 6 months pregnant, lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, 0-3 months pregnant, lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, not lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Female, pregnant, lactating</td>
<td>Female</td>
</tr>
<tr>
<td>Male, young bull</td>
<td>Male</td>
</tr>
<tr>
<td>Male, veal calf</td>
<td>Male</td>
</tr>
<tr>
<td>Female, heifer, 15-18 months old</td>
<td>Female</td>
</tr>
<tr>
<td>Male, fattening bulls *</td>
<td>Pregnenolone</td>
</tr>
<tr>
<td>Male, fattening bulls *</td>
<td>Male</td>
</tr>
<tr>
<td>Male, fattening bulls *</td>
<td>Male</td>
</tr>
<tr>
<td>Male, 24 months olds</td>
<td>Male</td>
</tr>
<tr>
<td>Female, 3-4 years old</td>
<td>Female</td>
</tr>
<tr>
<td>Female, 6 months old</td>
<td>Female</td>
</tr>
</tbody>
</table>

* 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)