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Michel Nielen, Toine Frank Henk Bovee, Henri Heskamp, Ron Laurentius Hoogenboom

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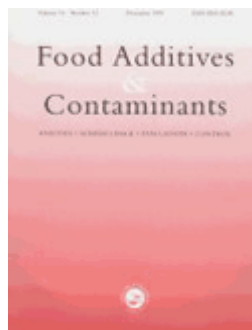
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Screening for estrogen residues in calf urine: comparison of a validated yeast estrogen bioassay and gas chromatography tandem mass spectrometry

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6 1 **Screening for estrogen residues in calf urine: comparison of a**
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9 2 **validated yeast estrogen bioassay and gas chromatography**
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11 3 **tandem mass spectrometry**
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15 5 M.W.F. NIELEN*, T.F.H. BOVEE, H.H. HESKAMP, J.J.P. LASAROMS, M.B. SANDERS, J.A.

16 6 VAN RHIJN, M.J. GROOT & L.A.P. HOOGENBOOM.

17 7 RIKILT Institute of Food Safety, P.O.Box 230, 6700 AE Wageningen, The Netherlands
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23 9 *Correspondence: M.W.F. Nielen. E-mail: michel.nielen@wur.nl
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1 Abstract

2 Within the European Union the control for residues of illegal hormones in food producing
3 animals is based on urine analysis for a few target analytes using gas chromatography mass
4 spectrometry and/or liquid chromatography tandem mass spectrometry. Recently, we developed
5 a robust yeast bioassay screening tool for estrogens which was validated as a qualitative
6 screening method in accordance with EC decision 2002/657/EC. In this study we present long-
7 term performance data and a comparison of urine data as obtained with this bioassay, and data
8 from an established gas chromatography tandem mass spectrometry (GC/MS/MS) confirmatory
9 analysis method. More than 120 calf urine samples from a controlled reference experiment were
10 analysed using both protocols. According to the GC/MS/MS method only the natural estrogens
11 17α -estradiol and estrone were present in the non-compliant samples. The bioassay was less
12 sensitive than GC/MS/MS for the relatively weak estrogenic compound 17α -estradiol, in
13 accordance with expectations. Assuming that application of the mass spectrometric method is
14 considered beyond reasonable doubt, the bioassay performed very well: only 5.6 % of the calf
15 urine samples found compliant in GC/MS/MS were screened false suspect in the bioassay
16 screening method. The bioassay results of non-compliant urine samples under routine
17 conditions were as predicted, taking into account the relative estrogenicity of the natural
18 estrogens 17α -estradiol and estrone versus 17β -estradiol. Only one sample was screened false
19 negative for 17α -estradiol and estrone. Application of this fast and simple estrogen bioassay in
20 routine surveillance and control can significantly reduce GC/MS/MS sample workload and allow
21 higher percentages of animals to be screened for potential hormone abuse.

22
23 **Keywords:** *Bioassay, estrogen, urine, screening, GC/MS/MS, residue analysis, steroid*

1

2 **Introduction**

3 The use of growth promoters for fattening purposes has been banned in the European Union
4 (EU) since 1988 (European Commission, 1996a). Residue analysis of target compounds is
5 carried out by several control laboratories in order to protect the consumer, guarantee fair trade,
6 and to enforce the ban (European Commission, 1996b). Many analyses are carried out in the
7 early stage of the food production chain, in urine and feed samples collected at the farms. The
8 EU regulation as laid down in 96/22/EC (European Commission, 1996a) prohibits the use of
9 substances having *hormonal activity* and β -agonists. Interestingly, no black-list of substances
10 but any substance having hormonal activity and any beta-agonist is prohibited. Consequently,
11 targeted gas chromatography/mass spectrometry (GC/MS) and liquid chromatography tandem
12 mass spectrometry (LC/MS/MS) methods, which are set-up to the monitoring of a few selected
13 ions or MS/MS transitions, can fulfill the control only to a limited extend (Hewitt et al. 2002; Van
14 Poucke et al. 2002; O'Keeffe et al. 2003; Impens et al. 2003). Moreover, residues of designer
15 steroids and β -agonists will escape from residue control until the original illicit preparations are
16 found, identified by NMR and mass spectrometry, and finally included in the targeted residue
17 analysis methods (Catlin et al. 2004; Nielen et al. 2003).

18

19 Recently we presented a new approach to the search for residues of estrogens in urine, based
20 on a robust yeast reporter gene bioassay and hormonal activity measurement (Bovee et al.
21 2004a, Bovee et al. 2004b). This yeast cell stably expresses the human estrogen alpha receptor
22 (hER α) and yeast enhanced green fluorescent protein (yEGFP) upon exposure to estrogens. In
23 contrast to receptor binding assays, reporter gene bioassays also mimic the transactivation step
24 and can distinguish between receptor agonists and receptor antagonists (Mueller, 2002).
25 Transcription activation bioassays based on human cell lines might be considered even more
26 relevant from a food safety perspective and are often even more sensitive than yeast based
27 bioassays (Sonneveld et al. 2005). However, yeast cells show distinct advantages since they do
28 not produce any steroid receptors until they have been modified to do so. Furthermore yeast
29 cell cultures do not require growth media such as calf serum which might contain steroids. Last

1 but not least, yeast cell assays are extremely robust and survive extracts from dirty sample
2 matrices such as urine and feed. Thus robust group-selective bioassays can be constructed
3 which respond selectively to a steroid family such as estrogens while still having good
4 sensitivity. The application of the estrogen bioassay to real urine (or feed) samples is very
5 simple and requires only the addition of the yeast suspension to the sample extract in a 96 well
6 plate, a 4 or 24 h incubation time and measurement in a plate reader. No reagent or cell lysis is
7 required, i.e. measurement at different time points might be easily accomplished, when
8 required. This bioassay is routinely applied as a qualitative screening method in both calf urine
9 and feed sample matrices: only sample extracts showing a fluorescence response beyond the
10 decision limit CC_{α} will be declared suspect for estrogen activity. Subsequently the identity of
11 substances causing the estrogenicity must be determined by conventional residue analysis
12 methods such as GC/MS or using bioassay-directed quadrupole time-of-flight MS (QTOFMS)
13 identification approaches (Nielen et al. 2004; Nielen et al. 2006). The sensitivity of the bioassay
14 is fit for purpose: the decision limit CC_{α} in response units as determined in the initial validation
15 study corresponds with as little as 0.2 ng/ml 17β -estradiol equivalents in calf urine. It is fair to
16 say that this sensitivity is dependent on the relative estrogenic potency, i.e. weaker estrogens
17 than 17β -estradiol such as for example the mycotoxin zearalenone are screened less sensitive.
18 On the other hand, weaker estrogens are less relevant from a hormonal activity point of view
19 and would require relatively high doses anyway. Unfortunately, official residue surveillance plans
20 (European Commission 1996b) within the EU do not differentiate yet between weak and strong
21 hormonal substances; they should be all measured at the more or less equal minimum required
22 performance level (MRPL) of 1-2 ng/ml. Nevertheless, the estrogen bioassay has been fully
23 validated for calf urine (Bovee et al. 2005) and feed samples against the latest EU guidelines
24 (European Commission 2002) and acquired recently an ISO17025 accreditation status in The
25 Netherlands.

26

27 The estrogen bioassay is being used for more than two years now. In this study we present the
28 long-term results from the control urine samples over that period and make a direct comparison

1 of the estrogen bioassay and an established GC/MS/MS method for the confirmatory analysis of
2 calf urine samples.

3

4 **Materials and methods**

5

6 *Reagents*

7 Water was purified using a Millipore (Bedford, MA, USA) model Milli-Q Gradient A10 system.
8 Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). The *suc* Helix
9 Pomatia β -glucuronidase/arylsulfatase, estrone, ammonium sulphate, dimethyl sulfoxide,
10 sodium acetate and sodium carbonate were obtained from Merck (Darmstadt, Germany).
11 Dithiothreitol, ammonium iodide, 17β -estradiol and 17α -estradiol were purchased from Sigma
12 (St. Louis, MO, USA). 17β -Estradiol-16,16,17-d₃ was from C/D/N Isotopes (Pointe-Claire,
13 Canada). N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Pierce
14 (Rockford, IL, USA). Isolute NH₂ solid phase extraction columns (100 mg) were from IST
15 (Hengoed, U.K.) and Bond Elut C18 solid phase extraction columns (500 mg) from Varian
16 (Harbor City, CA, USA). Dextrose and yeast nitrogen base without amino acids and without
17 ammonium sulphate were obtained from Difco (Detroit, MI, USA). The minimal cell exposure
18 medium with L-leucine medium (MM/L) consisted of yeast nitrogen base (1.7 g/l), dextrose (20
19 g/l) and ammonium sulphate (5 g/l) and was supplemented with L-leucine (60 mg/l).

20

21 *Samples*

22 Urine samples were obtained from a life-time controlled veal calve reference experiment. In
23 short, 21 male and 21 female calves were purchased at the age of 1-3 weeks, raised under
24 representative zootechnical conditions and slaughtered after 26 weeks. The animals were fed
25 via an automated milk system using regular milk replacer. According to histopathology of the
26 prostates and the Bartholin glands at slaughter, no intentional (or unintentional) hormone
27 treatment was detected (Groot et al. 2006). 126 urine samples were random collected during
28 the experiment at the age between 9 and 26 weeks and analysed by both the bioassay and the
29 GC/MS/MS protocols.

1

2 *Bioassay procedure*

3 Aliquots of 2 ml of sodium acetate reagent blank, blank calf urine, blank calf urine fortified with
4 17β -estradiol (1 ng/ml), and the samples under investigation, were adjusted to pH 4.8 and 20 μ l
5 β -glucuronidase/arylsulfatase (30 and 60 U/ml, respectively) was added. Enzymatic
6 deconjugation was carried out overnight in a water bath at 37 °C. Next, 2 ml of 0.25 M sodium
7 acetate buffer pH 4.8 was added and the hydrolysed sample was subjected to solid phase
8 extraction (SPE) on a C18 column fitted on a vacuum manifold. This column was previously
9 conditioned with 2.5 ml methanol and 2.5 ml sodium acetate buffer. Subsequently, this column
10 was washed with 1.5 ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5 ml sodium
11 acetate buffer pH 4.8, 3.0 ml water and finally with 2 ml methanol/water (50/50 v/v). The column
12 was air-dried and eluted with 4 ml acetonitrile, which was applied to an NH_2 -column, previously
13 conditioned with 3.0 ml acetonitrile. The acetonitrile eluate thus obtained was evaporated to 2
14 ml under a stream of nitrogen gas. A 100 μ l part of this extract (equivalent to 100 μ l urine) was
15 transferred to a 96 well plate in triplicate and 50 μ l water and 2 μ l DMSO were added to each
16 well. In order to remove the acetonitrile from this mixture, the plate was dried overnight in a
17 fume cupboard and was then ready to be screened on estrogenic activities with the yeast
18 estrogen bioassay.

19

20 The yeast cytosensor expressing the human estrogen receptor α (hER α) and yeast enhanced
21 green fluorescent protein (yEGFP) in response to estrogens was developed in-house and
22 described previously (Bovee et al. 2004a, Bovee et al. 2004b). In short, an agar plate containing
23 the selective MM/L medium was inoculated with the yeast ER α cytosensor from a frozen -80 °C
24 stock (20% glycerol v/v). The plate was incubated at 30 °C for 24-48 h and then stored at 4 °C.
25 The day before running the assay, a single colony of the yeast cytosensor was used to inoculate
26 10 ml of selective MM/L medium. This culture was grown overnight at 30 °C with vigorous orbital
27 shaking at 225 rpm. At the late log phase, the yeast ER α cytosensor was diluted (1:10) in MM/L.
28 For exposure in 96 well plates, aliquots of 200 μ l of this diluted yeast culture were pipetted into

1 each well, already containing the extracts of the urine samples. A 17β -estradiol dose-response
2 curve in DMSO was included in each exposure experiment. Each urine sample extract and each
3 17β -estradiol stock were assayed in triplicate. Exposure was performed for 0 h and 24 h.
4 Fluorescence at these time intervals was measured directly in a PerSeptive Biosystems
5 (Framingham, MA, USA) model CytoFluor Series 4000 multi-well plate reader using excitation at
6 485 nm and emission measurement at 530 nm. The $t_{24}-t_0$ differences (mean of the triplicates)
7 were calculated and corrected for the reagent blank data, thus providing the final estrogenic
8 activity data for each sample. The samples are reported "suspect" when the $t_{24}-t_0$ fluorescence
9 measurement exceeds the $CC\alpha$ as determined in the initial validation study (Bovee et al. 2005);
10 otherwise the samples are reported "compliant". The bioassay is a qualitative screening method,
11 i.e. no concentrations are reported. The densities of the yeast culture at the t_0 and t_{24} time
12 intervals are also determined by measuring the OD at 630 nm. This is done from a
13 precautionary principle to check whether a urine sample matrix is toxic for yeast. In routine
14 practice however such cell toxicity never occurred over the last two years.

15 *GC/MS/MS procedure*

16 Aliquots of 2 ml of sodium acetate reagent blank, blank calf urine, blank calf urine fortified with
17 17β -estradiol and other estrogens at 0.5, 1.0, 2.0, 5.0 and 10 ng/ml, and the urine samples
18 under investigation, were spiked with deuterium-labelled 17β -estradiol as internal standard at 2
19 ng/ml. The samples were enzymatically deconjugated and subjected to solid phase extraction
20 as described in the bioassay procedure, except for the elution solvent of the C18 and NH_2
21 extraction columns which was methanol instead of acetonitrile. The extracts thus obtained were
22 redissolved and fractionated by preparative reversed phase gradient LC on a 250 x 3.0 mm I.D.
23 Lichrocart (Merck) column packed with 5 μ m C18 RP-Select B material and equipped with a
24 Lichrocart 4-4 guard column packed with similar material. The two mobile phases used consisted
25 of (A) methanol/water (10:90) and (B) methanol/water (90:10) and the flow was 0.4 ml/min.
26 Following an isocratic period of 10 min at 65% B a steep linear gradient was started towards
27 95% B at 12 min, followed by a final isocratic step of another 9 min. The steroid fraction was
28 evaporated to dryness under a stream of nitrogen gas at 45 °C, redissolved in dry ethyl acetate,
29

1 evaporated and derivatised using so-called MSTFA++ reagent (a mixture of dithiothreitol,
2 ammonium iodide and MSTFA) for 15 minutes at 60 °C. Finally 2 µl of the silylated steroids
3 were injected into a GC/MS/MS system consisting of an Agilent (Palo Alto, CA, USA) model
4 6890N capillary gas chromatograph equipped with a splitless injector, helium as carrier gas, and
5 a 30 m x 0.25 mm I.D. Restek (Bad Homburg, Germany) model Rtx-CL Pesticides (df 0.25 µm)
6 column, coupled with a Micromass (Manchester, UK) model Quattro Micro GC/MS/MS system.
7 Following an initial temperature of 130 °C for two minutes, the GC oven was programmed
8 towards 250 °C at a rate of 12 °C/min, followed after 3 min by a second ramp towards 300 °C at a
9 rate of 7.5 °C/min. The total analysis time per sample injection was 35 min. The mass
10 spectrometer was operated in the Electron Impact (EI) ionisation mode at 70 eV, using an
11 interface temperature of 275 °C, a source temperature of 180 °C, argon (2.8×10^{-3} mbar) as CID
12 gas, and a solvent delay time of 8 min. The MRM acquisition parameters of the GC/MS/MS
13 protocol applied are given in Table I. Depending on specific interests of the veterinary inspectors
14 this multi-residue GC/MS/MS method can be easily extended to include other steroids and
15 macrocyclic resorcylic lactones.

16
17 “[Insert Table I about here]”
18

19 Calf urine samples were positively confirmed and reported *non-compliant* when the GC retention
20 time and MS/MS ion ratio criteria were fulfilled (European Commission 2002). Concentrations
21 were calculated and corrected for recovery losses using the isotope dilution method and a
22 calibration curve based on matrix-matched standards, i.e. blank urine samples spiked with 0, 0.5,
23 1.0, 2.0, 5.0 and 10 ng/ml of a steroid mixture.

24 25 **Results and discussion**

26 27 *Routine performance of the estrogen bioassay*

28 A general concern when applying bioassays for screening is the occurrence of biologically
29 inactivated substances in urine matrices. However in the experimental procedure inactivated

1 phase II metabolites are reconverted into their corresponding phase I metabolites, thanks to the
2 enzymatic sample pretreatment step. In a previous study [Bovee et al. 2004b] it was shown that
3 phase I metabolites such as 17α -estradiol, estrone, estriol and hydroxylated estrogens do give
4 a response in the bioassay, but they require a higher concentration for detection. In hormone
5 treatment of cattle the urinary profile usually contains a mixture of phase II metabolites of the
6 parent drug and its phase I metabolites. Since the bioassay provides an additive measurement
7 of alle estrogenic residues and metabolites (having different relative estrogenic potencies) the
8 chance of overlooking illegal treatment is relatively low as compared to other screening assays
9 such as highly specific immuno assays. The estrogen bioassay is routinely applied in our
10 laboratory for the screening of urine samples for more than two years now. Cell toxicity caused
11 by matrix components in the urine extract was never observed. A control chart is given in Figure
12 1.

13 “[Insert Figure 1 about here]”

14
15 From this chart it can be seen that negative reagent blank and calf urine control samples were
16 compliant during this period, except for two blank urine cases which were false suspect (slightly
17 above $CC\alpha$). The positive calf urine control samples (blank urines fortified with 1 ng/ml 17β -
18 estradiol) were always found suspect in the same period, i.e. false compliant results were not
19 observed at all. Despite the inherent variability caused by variable analyte recovery and natural
20 fluctuations in the biological assay, the data presented here clearly show that application of
21 hormone bioassays in routine control is feasible, even on the long-term, provided that the assay
22 is applied as an on/off qualitative screening method rather than a quantitative assay. These
23 results from long-term routine practice are in accordance with expectations from the initial
24 validation study (Bovee et al., 2005). Moreover, these findings support the ruggedness and
25 stability of the entire assay including sample preparation, cell culturing, cell growth and
26 detection; otherwise the control sample results would not be so consistent over such a long time
27 period.

28

29 *Comparison between the bioassay and GC/MS/MS results*

1 GC/MS(/MS) is the routine screening and confirmatory analysis method for steroids in urine
2 samples in many veterinary residue control and sports doping laboratories worldwide. Typically,
3 following a liquid-liquid or solid phase extraction protocol the extracts are fractionated, silylated
4 and analysed. The performance characteristics of the GC/MS/MS versus the bioassay
5 screening of natural estrogens in urine are given in Table II.

6 “[Insert Table II about here]”

7
8 In this study we assumed the GC/MS/MS confirmatory analysis method to perform beyond
9 reasonable doubt, which is justified by the precision and accuracy data given in Table II, and
10 made a comparison with the yeast estrogen bioassay screening method. 126 calf urine samples
11 collected during a controlled reference experiment at different time points between 9 and 26
12 weeks were subjected to both the bioassay and the GC/MS/MS protocols. The results of both
13 methods are summarised in Figure 2. Again, none of the urine samples caused any cell toxicity
14 in the bioassay, in accordance with our two years experience with this estrogen bioassay. The
15 negative urine bioassay controls were compliant and the positive (1 ng/ml 17 β -estradiol) urine
16 controls were screened “suspect” in all bioassay series. In the GC/MS/MS series the negative
17 controls were compliant and the fortified urine control samples were always confirmed as non-
18 compliant according to the EU criteria (European Commission 2002). According to the
19 GC/MS/MS method 71 out of the 126 calf urine samples were compliant for estrogens, i.e. all
20 estrogens included in this instrumental multi-residue method were below 1 ng/ml.

21
22 “[Insert Figure 2 about here]”

23
24 In the bioassay screening 67 out of 126 were screened compliant for estrogen activity. Only 4
25 out of 71 (5.6 %) might be considered false-suspects in comparison with the GC/MS/MS results,
26 but it should be kept in mind that these four urine samples might really contain (a mixture of) low
27 levels of estrogens because of the additive bioactivity measurement. No attempts were made to
28 identify this mixture of low levels of estrogens since in a controlled reference experiment no
29 illegal treatment with synthetic estrogens is expected anyway.

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6 2 In the GC/MS/MS method 55 urine samples were found to contain estrogens at a level of 1
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8 3 ng/ml or more. In all cases the natural estrogen 17α -estradiol was identified (concentrations are
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10 4 given in Figure 2), occasionally accompanied by estrone. The presence of these natural
11
12 5 estrogens in urine from individual calves is not unexpected when they reach the age of 20-28
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14 6 weeks.

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18 8 For a fair comparison of the non-compliant samples found by the quantitative GC/MS/MS
19
20 9 confirmatory analysis method and the suspect samples found by the qualitative bioassay
21
22 10 screening method, one should consider the sensitivity of the bioassay for these estrogens.
23
24 11 Bioassay response curves for 17α -estradiol and estrone were recorded in triplicate and are
25
26 12 shown in Figure 3, together with the reference curve for 17β -estradiol.

27
28 13 “[Insert Figure 3 about here]”
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31
32 15 The results are in good agreement with the previous literature data: the sensitivity of the
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34 16 estrogen bioassay is dependent on the estrogenic potencies of the analytes present. According
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36 17 to literature the relative estrogenic potency (REP) of 17α -estradiol and estrone versus 17β -
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38 18 estradiol is 0.09 and 0.2, respectively (Bovee et al., 2004b), similar to the data in Figure 3. The
39
40 19 decision limit $CC\alpha$ and the detection capability $CC\beta$ of the bioassay were determined in the
41
42 20 validation study (Bovee et al., 2005), but for 17β -estradiol and *not* for the natural estrogens 17α -
43
44 21 estradiol and estrone (cf. Table II). However, the $CC\alpha_{17\alpha\text{-estradiol}}$, $CC\beta_{17\alpha\text{-estradiol}}$ (and $CC\alpha_{\text{estrone}}$
45
46 22 and $CC\beta_{\text{estrone}}$) can be predicted from the 17β -estradiol data and the REP's, as follows: the
47
48 23 $CC\alpha_{17\beta\text{-estradiol}}$ in fluorescence response units corresponds with 0.22 ng 17β -estradiol equivalents
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50 24 per ml (Bovee et al., 2005). The $CC\beta_{17\beta\text{-estradiol}}$ as calculated from this $CC\alpha$ was 0.44 ng/ml, while
51
52 25 the experimentally verified $CC\beta_{17\beta\text{-estradiol}}$ was < 1 ng 17β -estradiol equivalents per ml (Bovee et
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54 26 al., 2005). When these $CC\beta$ and $CC\alpha$ values of 17β -estradiol are divided by the REP-values of
55
56 27 17α -estradiol and estrone (Bovee et al., 2004b), a prediction of the $CC\alpha$ and $CC\beta$ is obtained
57
58 28 for 17α -estradiol and estrone. By doing so a bioassay response might be expected for 17α -
59
60

1 estradiol in urine starting from 2.4 ng/ml ($CC_{\alpha_{17\alpha\text{-estradiol}}}$) and for estrone starting from 1.1 ng/ml
2 ($CC_{\alpha_{\text{estrone}}}$). Indeed from the 38 GC/MS/MS results containing 1 to 3 ng/ml 17α -estradiol only a
3 few samples were screened suspect in the bioassay. Beyond 3 ng/ml 17α -estradiol in urine one
4 might expect more consistency between GC/MS/MS and bioassay data: at and beyond the
5 predicted CC_{β} of 17α -estradiol, i.e. above 5-11 ng/ml (depending on whether the calculated or
6 experimentally determined $CC_{\beta_{17\beta\text{-estradiol}}}$ has been used for the prediction) at least 95%
7 consistency might be expected provided the CC_{β} data of 17β -estradiol from the bioassay
8 validation study (Bovee et al. 2005) and the REP data of 17α -estradiol (Bovee et al. 2004b) are
9 valid and, moreover, provided the recovery is extremely reproducible. According to Figure 2 this
10 expectation is valid except for two urine samples, one containing 5.5 ng/ml 17α -estradiol
11 (rounded off to 6 ng/ml in Figure 2) and a second one containing 10 ng/ml 17α -estradiol by
12 GC/MS/MS. The discrepancy observed between the GC/MS/MS and the bioassay data at the
13 5.5 ng/ml level can be easily explained when one takes into account that the concentration as
14 determined by the GC/MS/MS method was corrected for incomplete recovery using deuterium-
15 labelled estradiol as internal standard. It should be noted that the predicted CC_{β} of 17α -
16 estradiol ranges from 5 to < 11 ng/ml, depending on whether the calculated or experimentally
17 verified CC_{β} of 17β -estradiol was taken; only the experimentally determined value includes
18 recovery losses. The bioassay did show increased fluorescence for the 5.5 ng/ml 17α -estradiol
19 sample but the level was just below the CC_{α} threshold for being qualified as "suspect".
20 According to GC/MS/MS the urine sample containing 10 ng/ml 17α -estradiol also contained a
21 low level of estrone which should have triggered the bioassay in addition to the 17α -estradiol
22 level. The GC/MS/MS reconstructed chromatograms shown in Figure 4 clearly indicate the
23 presence of these compounds in this particular urine sample.

24 "[Insert Figure 4 about here]"

25

26 The estrogen bioassay did not show any increased fluorescence so the bioassay result for this
27 specific sample suggests a real false-compliant case (1 out of a total of 126 samples). Apart
28 from a human error such as sample interchange no other major discrepancy sources are to be

1 expected since the sample preparation is quite similar for both methods. The original incurred
2 urine sample was re-analysed by both the bioassay and the GC/MS/MS in 6-fold, but nine
3 months later. In all six replicates the bioassay showed a strong signal and the sample was
4 screened suspect without any doubt and in full agreement with the 10 ng/ml GC/MS/MS data,
5 even after 9 months. Obviously the stability of calf urine samples under appropriate storage
6 conditions is much longer than the ≥ 90 days determined in the validation study (Bovee et al.
7 2005). From the results of the re-analysis it was concluded that the apparent false-negative
8 urine sample was most likely caused by a human error.

9
10 Currently, the bioassay screening is limited to calf urine: older bovines produce quite a lot of
11 endogenous 17α -estradiol and estrone and will cause too many false suspect urine data. We
12 are currently investigating the option of selective removal of 17α -estradiol and estrone from
13 bovine urine in order to extend the scope of this method to older animals.

14 15 **Conclusion**

16
17 The routine performance of a previously validated estrogen bioassay is in full accordance with
18 expectations. The results of negative and positive calf urine control samples analysed in a
19 period covering more than two years indicate a very good ruggedness over time. In a direct
20 comparison with an established GC/MS/MS steroid analysis method the estrogen bioassay
21 showed a low percentage (5.6%) of potential false suspect results. The bioassay screening can
22 be considered fast and simple: plates with yeast cells are stable at 4 °C for over one month and
23 can be used directly to inoculate a culture for testing. The next day the culture is ready to be
24 used for screening of the samples, thus requiring some planning the day prior to analysis.
25 Samples can be screened in parallel using a 96-well plate and, without the addition of any
26 reagent, fluorescence is measured by a simple plate reader after 4 or 24 hours. It is true that
27 recombinant yeast requires special laboratory facilities, but for this recombinant yeast only an
28 ML1- category biohazard laboratory is needed. The costs of bioassay screening are so low that
29 one can even apply it as an HPLC detector (Nielen et al. 2004; Nielen et al. 2006). Thus

1 screening with the estrogen bioassay will significantly reduce the sample workload of the
2 instrumental GC/MS/MS analysis method.

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4 In the samples found non-compliant by GC/MS/MS the bioassay responded as predicted on the
5 basis of the relative estrogenic potency of the analytes involved. Indeed the bioassay is less
6 sensitive for weak estrogens than GC/MS/MS, but perhaps that is more relevant than findings of
7 very low levels of inactive substances. After all, the legislative fundament laid down in 96/22/EC
8 (European Commission, 1996a) bans substances having hormonal activity.

9 10 **Acknowledgements**

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15 Table I. MS/MS conditions of the MRM acquisition used in the GC/MS/MS analysis of TMS
16 derivatised calf urine extracts.

Component	Ion transition for screening (m/z)	Ion transition for confirmation (m/z)	Collision energy (eV)
Hexestrol	207 > 179	207 > 163	10
Dienestrol	410 > 395	410 > 179	10
<i>cis</i> -Diethylstilbestrol	412 > 383	412 > 217	15
<i>trans</i> -Diethylstilbestrol	412 > 383	412 > 217	15
Ethylestrenol	270 > 241	241 > 145	15
Estrane(5a)-3b,17a-diol	332 > 201	332 > 291	5
Equol	386 > 192	386 > 267	10
17a-Nortestosterone	418 > 194	418 > 182	15
5b-Androstane-17a-methyl-3a,17b-diol	270 > 255	435 > 345	10
Estrone	414 > 155	399 > 155	15
17a-Estradiol	416 > 285	416 > 326	12
17b-Nortestosterone	418 > 194	418 > 182	15
17b-Nortestosterone-d3 (ISTD)	421 > 182	-	15
17a-Testosterone	432 > 209	432 > 133	15
Methandriol	253 > 197	268 > 253	15

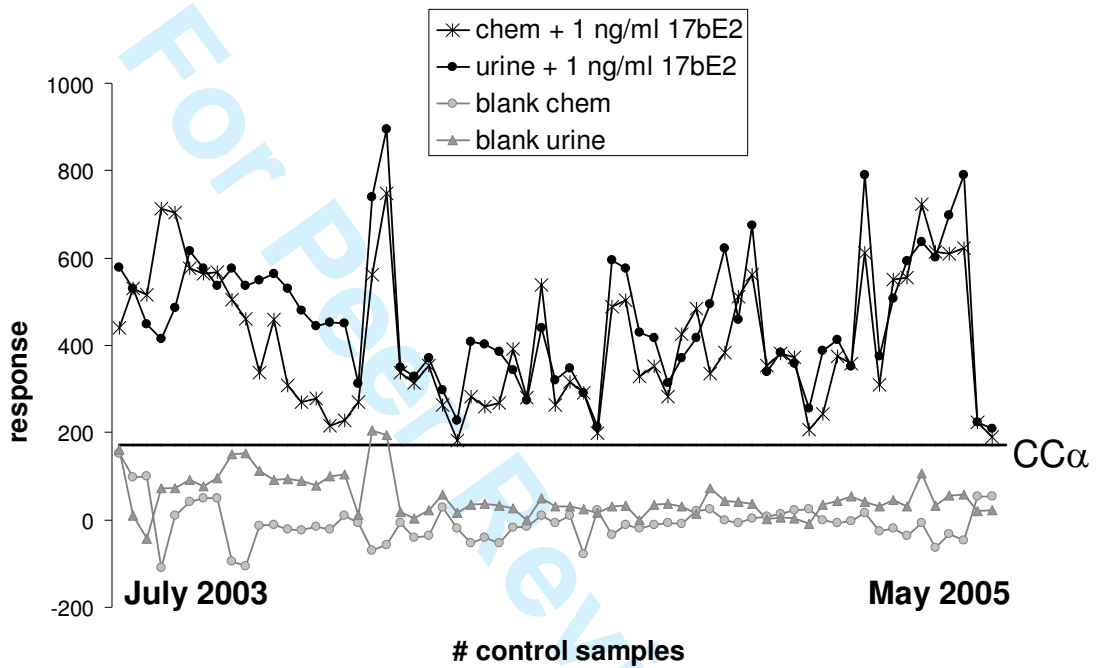
17b-Estradiol	416 > 285	416 > 326	12
17b-Estradiol-d3 (ISTD)	419 > 285	-	10
17a-Ethyl-5b-estrane-3a,17b-diol	421 > 331	421 > 241	10
17b-Testosterone	432 > 209	432 > 133	15
Methenolone	208 > 119	208 > 193	15
5a-Androstane-17a-methyl-3b,17b-diol	435 > 255	435 > 345	10
17a-Ethinylestradiol	425 > 193	300 > 232	15
17a-Methyltestosterone	446 > 301	301 > 169	12
17a-Methyltestosterone-d3 (ISTD)	449 > 301	-	10
Norethandrolone	446 > 287	287 > 155	10
Norgestrel	456 > 316	456 > 301	12
Chloroandrostedione	464 > 429	464 > 169	15
Fluoxymesterone	480 > 335	390 > 375	12

Table II. Performance characteristics of the analysis of natural estrogens in urine by GC/MS/MS versus the yeast estrogen bioassay data in literature (Bovee et al. 2005).

Component	LOD (ng/ml)	CC β (ng/ml)	Precision (%RSD) at 1.0 ng/ml (n=6)	Recovery (%) at 1.0 ng/ml	Accuracy (%) 17 β -estradiol-d ₃ corrected	specificity
17 β -estradiol						
-GC/MS/MS	0.5	< 1.0	7.1	27	90	passed
-Bioassay	0.22 ^a	< 1.0 ^b (0.44 ^c)	N.A.	N.A.	N.A.	passed
17 α -estradiol						
-GC/MS/MS	0.8	< 1.0	5.2	35	118	passed
-Bioassay	n.d.	n.d.	N.A.	N.A.	N.A.	n.d.
Estrone						
-GC/MS/MS	0.5	< 1.0	13	25	85	passed
-Bioassay	n.d.	n.d.	N.A.	N.A.	N.A.	n.d.

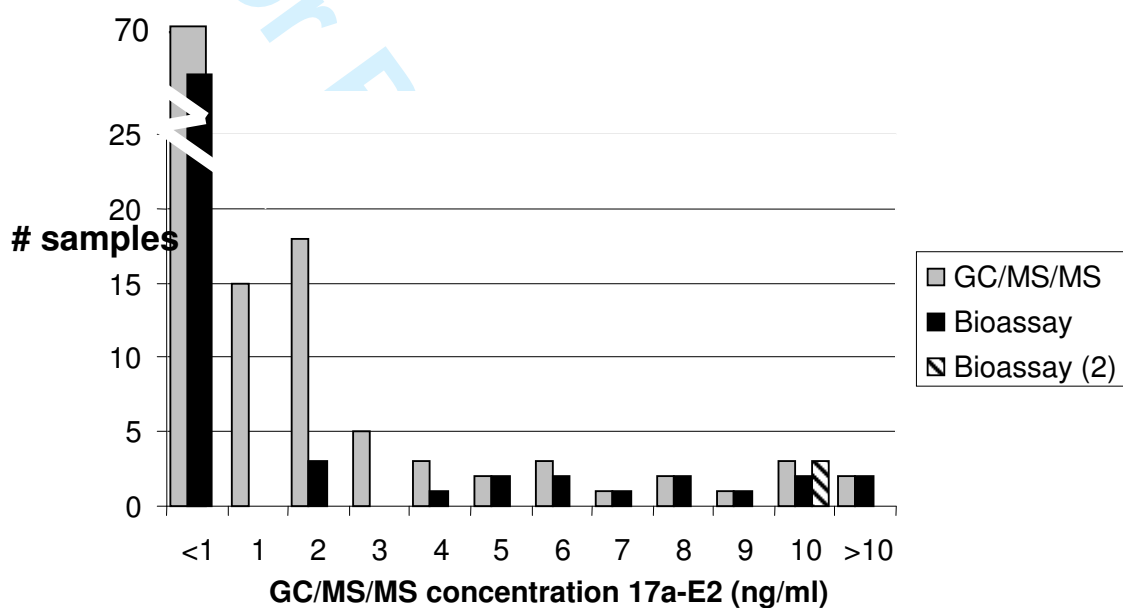
^a, CC α instead of LOD; ^b, experimentally determined: 20 out of 20 spiked urines were screened suspect at the 1.0 ng/ml level; ^c, calculated from the CC α value; N.A., not applicable; n.d., not determined

Figure 1. Control chart of the routine urine application of the bioassay, covering the period 2003-2005. In this period 63 sample series were analysed including reagent blanks, urine blanks, and both blanks spiked with 1 ng/ml 17 β -estradiol.



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2 Figure 2. Estrogen screening in calf urine samples: histogram comparison of the 17 α -estradiol (17a-E2) results obtained by a validated yeast Bioassay and by an established GC/MS/MS instrumental analysis method. The second bioassay data represent the re-analysis of a specific 10 ng/ml incurred urine sample, see text.

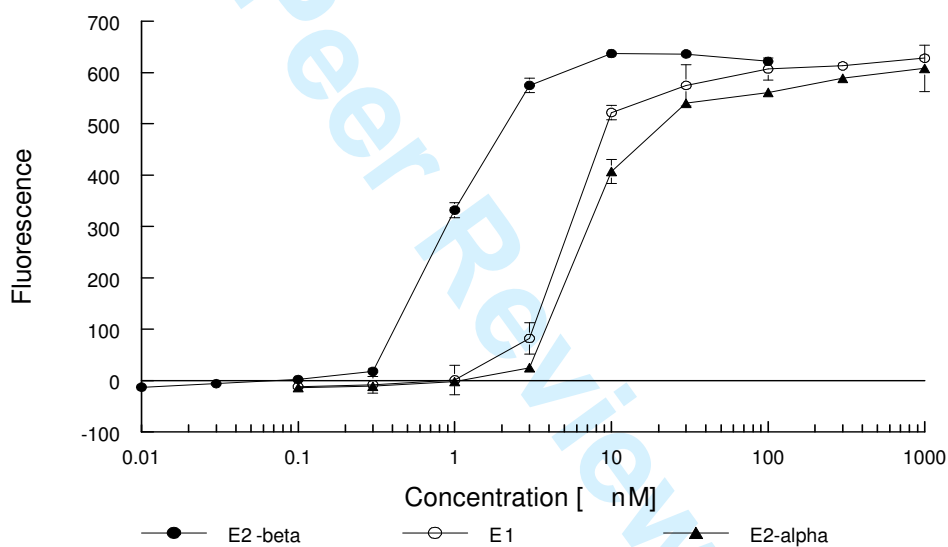


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2 Figure 3. Response curves of the yeast estrogen bioassay after a 24 h exposure to 17 β -
3 estradiol (E2-beta), 17 α -estradiol (E2-alpha) and estrone (E1). Measurements were performed
4 in triplicate (mean values and standard deviations are shown) and corrected for the signals
5 obtained at t_0 and the signal from a reagent blank exposure. For conditions, see Materials and
6 Methods.

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2 Figure 4. Reconstructed GC/MS/MS chromatograms of the urine sample containing
3 approximately 10 ng/ml of 17 α -estradiol. For conditions, see Materials and Methods and Table I.

