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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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## Abstract

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


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

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## Introduction

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

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

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

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

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

## Materials and methods

*Reagents*

Water was purified using a Millipore (Bedford, MA, USA) model Milli-Q Gradient A10 system. Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). The *suc* Helix Pomatia β-glucuronidase/arylsulfatase, estrone, ammonium sulphate, dimethyl sulfoxide, sodium acetate and sodium carbonate were obtained from Merck (Darmstadt, Germany). Dithiothreitol, ammonium iodide, 17β-estradiol and 17α-estradiol were purchased from Sigma (St. Louis, MO, USA). 17β-Estradiol-16,16,17-d3 was from C/D/N Isotopes (Pointe-Claire, Canada). N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was obtained from Pierce (Rockford, IL, USA). Isolute $NH_2$ solid phase extraction columns (100 mg) were from IST (Hengoed, U.K.) and Bond Elut C18 solid phase extraction columns (500 mg) from Varian (Harbor City, CA, USA). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Difco (Detroit, MI, USA). The minimal cell exposure medium with L-leucine medium (MM/L) consisted of yeast nitrogen base (1.7 g/l), dextrose (20 g/l) and ammonium sulphate (5 g/l) and was supplemented with L-leucine (60 mg/l).

*Samples*

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

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*Bioassay procedure*

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

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

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

*GC/MS/MS procedure*

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

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

"[Insert Table I about here]"

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

## Results and discussion

*Routine performance of the estrogen bioassay*

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

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

"[Insert Figure 1 about here]"

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

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

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

"[Insert Table II about here]"

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

"[Insert Figure 2 about here]"

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

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

For a fair comparison of the non-compliant samples found by the quantitative GC/MS/MS confirmatory analysis method and the suspect samples found by the qualitative bioassay screening method, one should consider the sensitvity of the bioassay for these estrogens. Bioassay response curves for 17α-estradiol and estrone were recorded in triplicate and are shown in Figure 3, together with the reference curve for 17β-estradiol.

"[Insert Figure 3 about here]"

The results are in good agreement with the previous literature data: the sensitivity of the estrogen bioassay is dependent on the estrogenic potencies of the analytes present. According to literature the relative estrogenic potency (REP) of 17α-estradiol and estrone versus 17β-estradiol is 0.09 and 0.2, respectively (Bovee et al., 2004b), similar to the data in Figure 3. The decision limit CCα and the detection capability CCβ of the bioassay were determined in the validation study (Bovee et al., 2005), but for 17β-estradiol and *not* for the natural estrogens 17α-estradiol and estrone (cf. Table II). However, the $CC\alpha_{17\alpha\text{-estradiol}}$, $CC\beta_{17\alpha\text{-estradiol}}$ (and $CC\alpha_{estrone}$ and $CC\beta_{estrone}$) can be predicted from the 17β-estradiol data and the REP's, as follows: the $CC\alpha_{17\beta\text{-estradiol}}$ in fluorescence response units corresponds with 0.22 ng 17β-estradiol equivalents per ml (Bovee et al., 2005). The $CC\beta_{17\beta\text{-estradiol}}$ as calculated from this CCα was 0.44 ng/ml, while the experimentally verified $CC\beta_{17\beta\text{-estradiol}}$ was < 1 ng 17β-estradiol equivalents per ml (Bovee et al., 2005). When these CCβ and CCα values of 17β-estradiol are divided by the REP-values of 17α-estradiol and estrone (Bovee et al., 2004b), a prediction of the CCα and CCβ is obtained for 17α-estradiol and estrone. By doing so a bioassay response might be expected for 17α-

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

“[Insert Figure 4 about here]”

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

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

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

**Conclusion**

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

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

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

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Table I. MS/MS conditions of the MRM acquisition used in the GC/MS/MS analysis of TMS 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 |
| *cis*-Diethylstilbestrol | 412 > 383 | 412 > 217 | 15 |
| *trans*-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 |
| Chloroandrostenedione | 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)** | **CCβ (ng/ml)** | **Precision (%RSD) at 1.0 ng/ml (n=6)** | **Recovery (%) at 1.0 ng/ml** | **Accuracy (%) 17β-estradiol-$d_3$ corrected** | **specificity** |
|---|---|---|---|---|---|---|
| 17β-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 |
| 17α-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], CCα instead of LOD; [b], experimentally determined: 20 out of 20 spiked urines were screened suspect at the 1.0 ngml level; [c], calculated from the CCα value;
N.A., not applicable; n.d., not determined

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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 17β-estradiol.

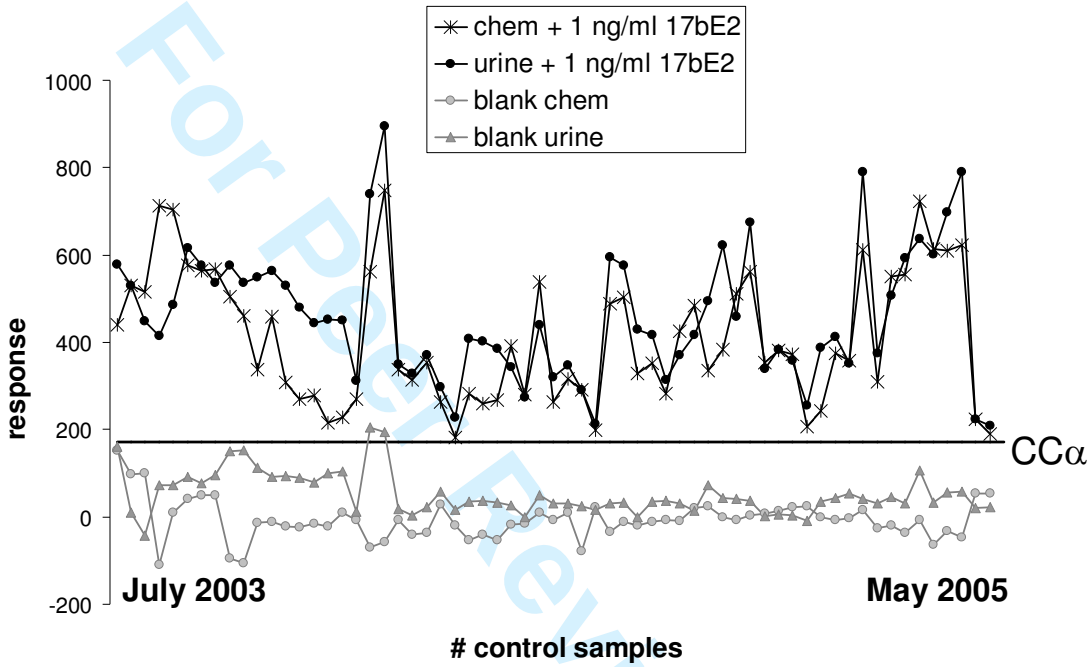


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Figure 2. Estrogen screening in calf urine samples: histogram comparison of the 17α-estradiol (17a-E2) results obtained by a validated yeast Bioassay and by an established GC/MS/MS instrumental analysis method. The second bioassay data represent the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

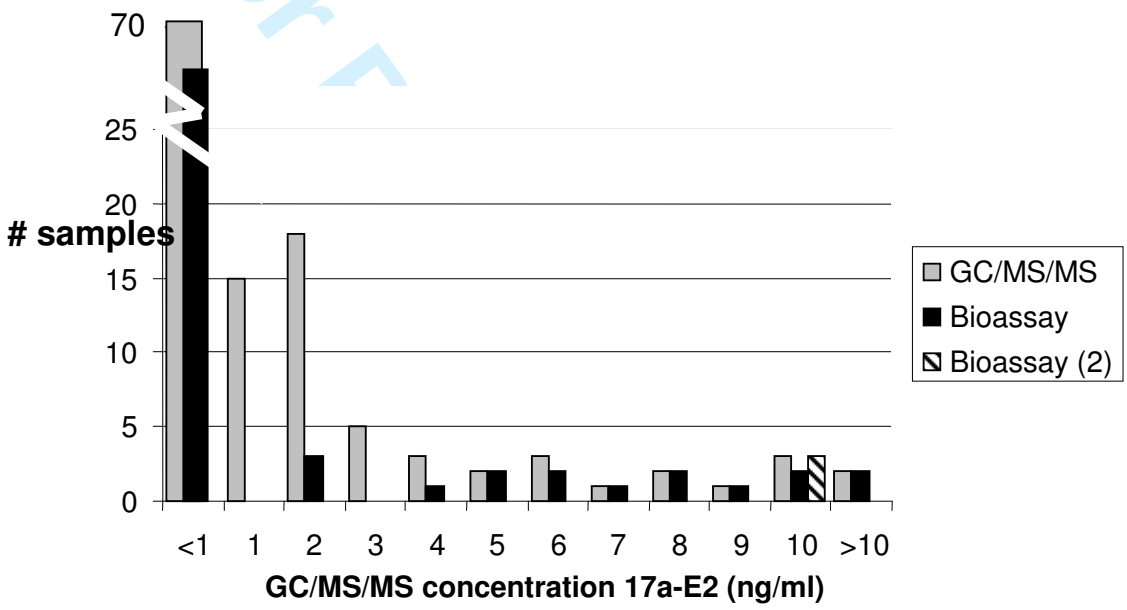


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Figure 3. Response curves of the yeast estrogen bioassay after a 24 h exposure to 17β-estradiol (E2-beta), 17α-estradiol (E2-alpha) and estrone (E1). Measurements were performed in triplicate (mean values and standard deviations are shown) and corrected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

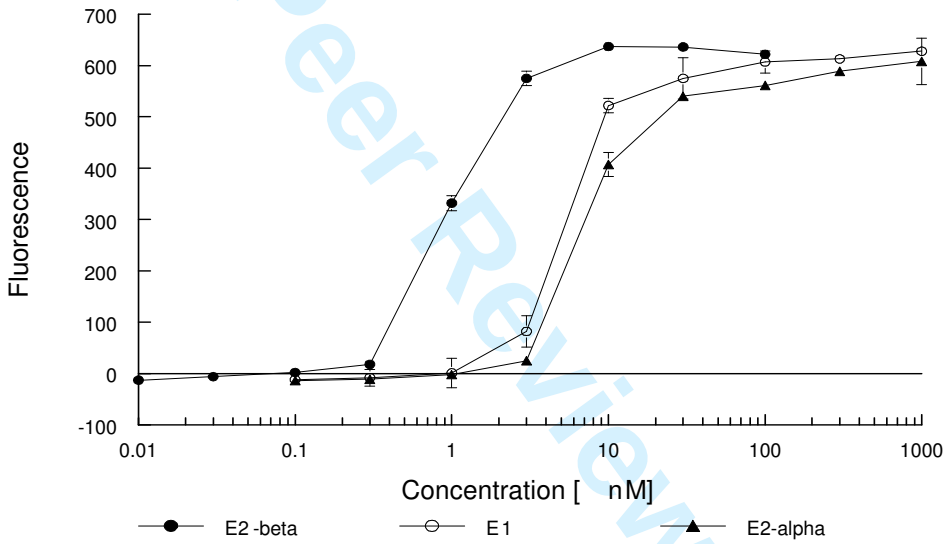


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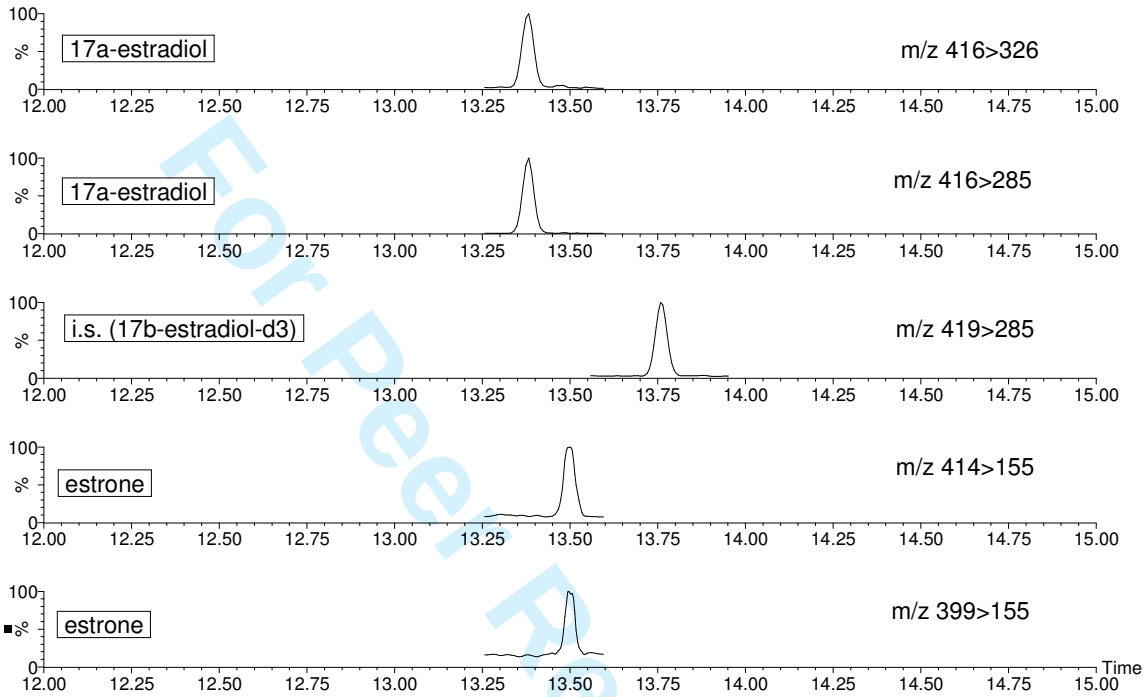
Figure 4. Reconstructed GC/MS/MS chromatograms of the urine sample containing approximately 10 ng/ml of 17α-estradiol. For conditions, see Materials and Methods and Table I.

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