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Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.	
Figure1-dose-response.TC	

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## Validation and application of a robust yeast estrogen bioassay for the screening of estrogenic activity in animal feed

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### Abstract

Previously we described the construction and properties of a rapid yeast bioassay stably expressing human estrogen receptor  $\alpha$  (hER $\alpha$ ) and yeast enhanced green fluorescent protein (yEGFP), the latter in response to estrogens. In the present study this yeast estrogen assay was validated as a qualitative screening method for the determination of estrogenic activity in animal feed. This validation was performed according to EC Decision 2002/657. Twenty blank animal feed samples, including milk replacers and wet and dry feed samples, were spiked with 17 $\beta$ -estradiol (E2 $\beta$ ) at 5 ng g<sup>-1</sup>, 17 $\alpha$ -ethynylestradiol (EE2) at 5 ng g<sup>-1</sup>, diethylstilbestrol (DES) at 10 ng g<sup>-1</sup>, zearalenone at 1.25  $\mu$ g g<sup>-1</sup> or equol at 200  $\mu$ g g<sup>-1</sup>. All of these blank and low estrogen spiked feed samples fulfilled the CC $\alpha$  and CC $\beta$  criterions, meaning that all 20 blank feed samples gave a signal below the determined decision limit CC $\alpha$  and were thus classified as compliant and at least 19 out of the 20 spiked samples gave a signal above this CC $\alpha$  ( $\beta$ =5%) and were thus classified as suspect. The method was specific and estrogens in feed were stable for up to 98 days. In this study we also present long-term performance data and several examples of estrogens found in the routine screening of animal feed.

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This is the first successful example of a developed, validated and applied bioassay for the screening of hormonal substances in feed.

Keywords: animal feed, estrogens, pharmaceutical waste, validation, yeast bioassay

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## 1 Introduction

Estrogens can be present in animal feed by inclusion of ingredients containing phytoestrogens but also by hormone abuse or even by incidents in which pharmaceutical waste is mixed into feed. The 2002 MPA-incident in the European Union showed that animal feed was contaminated with medroxyprogesterone 17-acetate (MPA), due to syrup that was contaminated with pharmaceutical waste (Van Leengoed et al., 2002). It should be emphasised that waste from pharmaceutical steroid production might contain estrogens as well, since the female birth control pill and tablets for hormone replacement therapy (HRT) both contain large amounts of estrogens. Estrogens are known to be used illegally to increase meat production, as it stimulates the growth of calves (Meyer et al., 1990; Guarda et al., 1990; Smith et al.). The use of growth promoters for fattening purposes in cattle has been banned in the European Union since 1988 (EC Council Directive 96/22). Countries outside the EU have a list of substances that are not prohibited or have no legislation at all. The EU ban prohibits all substances having hormonal action and does not provide a black-list of substances. In practice residue analysis, while aiming at consumer protection, fair trade and enforcement of the ban, is still carried out on specific target compounds (EC Council Directive 96/23) and can thus fulfil the control only to a limited extent. Histopathological effects in tissues can be examined and used to establish the use of growth promoting agents (Groot and Biolatti, 2004). However, these tests are only useful at the end of the food production, as they can not be used to test preparations, urine or animal feed samples. Thus they are not suited to be used as an early warning system. Alternatively, receptor based assays can be used to detect all compounds having affinity for a given receptor (Garcia-Reyero et al., 2000; Mueller, 2002; Michelini et al., 2004). 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). This feature is very helpful in detecting known and unknown compounds, as receptor stimulation plays a key role in the mechanism of action of growth promoters.

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3 Several assays have been developed for this purpose, using both mammalian and yeast  
4 cells. In general transcription activation assays based on mammalian or more particular  
5 human cell lines have been shown to be more sensitive than yeast based assays, and  
6 may be able to identify compounds that require human metabolism for activation into their  
7 active state (Legner et al., 1999; Hoogenboom et al., 2001; Sonneveld et al. 2005).  
8 However, yeast based assays have several other advantages. These include low costs  
9 and easier handling, lack of known endogenous receptors that may compete with the  
10 activity under investigation, use of media that are devoid of steroids (Breithofer et al.,  
11 1998; Graumann et al., 1999; Witters et al., 2001), and last but not least, yeast cells  
12 assays are extremely robust and survive extracts from dirty sample matrices such as  
13 urine (Bovee et al., 2005; Nielen et al., submitted). Recently we developed a novel yeast  
14 estrogen bioassay (REA), stably expressing human estrogen receptor  $\alpha$  (hER $\alpha$ ) and  
15 expressing yeast enhanced green fluorescent protein (yEGFP) in response to estrogens.  
16 The only handling required to perform this assay is the addition of the yeast suspension  
17 to a sample extract, followed by fluorescence measurement after 24 h. This assay is  
18 completely performed in a 96 well plate and fluorescence is measured directly in intact  
19 yeast cells in a plate reader. This assay is relatively simple and sensitive, as shown by an  
20 EC50 value for E2 $\beta$  of 0.7 nM (Bovee et al., 2004a). Furthermore, to investigate the  
21 specificity of the assay, a large number of chemically different compounds with known  
22 estrogenic, but also other hormonal activities were tested. All these estrogenic  
23 compounds caused a dose-related increase in the production of green fluorescent  
24 protein, whereas the gestagens progesterone and medroxyprogesterone 17-acetate  
25 showed no response and the androgen 17 $\beta$ -testosterone only showed a very weak  
26 response (Bovee et al., 2004b). This bioassay has been fully validated for calf urine  
27 (Bovee et al., 2005) and recently acquired an ISO17025 accreditation status in The  
28 Netherlands for this matrix.

29  
30 The present study describes the validation of the assay for animal feed. Regulation (EC)  
31 No 882/2004 on official controls performed to ensure the verification of compliance with  
32 feed and food law, animal health and animal welfare rules, that shall apply from 1 January  
33 2006, prescribes that laboratories involved in the analysis of official samples should work

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3 in accordance with internationally approved procedures or criteria-based performance  
4 standards and use methods of analysis that have, as far as possible, been validated.  
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6 Therefore, the yeast estrogen bioassay for the screening of estrogenic activity in animal  
7 feed was validated as the Commission Decision of 12 August 2002 of the EC prescribes  
8 in EC Decision 2002/657. As this decision prescribes that validation of a qualitative  
9 screening method must include verification of the detection capability ( $CC\beta$ ), specificity  
10 and stability, these performance characteristics are determined in the present study.  $17\beta$ -  
11 Estradiol ( $E2\beta$ ),  $17\alpha$ -ethynylestradiol (EE2), diethylstilbestrol (DES), zearalenone and  
12 equol were chosen as model compounds, because  $E2\beta$ , EE2 and DES are priority  
13 compounds for both hormone abuse and pharmaceutical waste and because of the  
14 differences in their polarity, with equol being the most polar and DES being the most a-  
15 polar compound. Furthermore, zearalenone and equol are natural compounds that may  
16 occur in animal feed. Zearalenone is an estrogenic mycotoxin and equol is an estrogenic  
17 metabolite of the less estrogenic phytoestrogen daidzein. As representatives for feed  
18 samples, 7 wet pulp feed samples (pigs), 7 dry grinded feed samples (ruminants, poultry,  
19 pigs) and 6 regular milk replacer samples (calves) were used. In this study we also  
20 present long-term performance data for the screening of estrogenic activity in animal  
21 feed. The applicability of this early warning bioassay system was further demonstrated by  
22 screening and identifying the estrogenic activity found in wet pulp feed samples of the  
23 MPA-incident and in the routine screening of fish feed samples.  
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## 43 44 45 **2. Experimental**

### 46 47 48 49 **2.1 Chemicals**

50 Water was purified using a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA).  
51 Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands).  
52 Ammonium sulphate, dimethyl sulfoxide, sodium acetate and sodium carbonate were  
53 obtained from Merck (Darmstadt, Germany). The compounds  $17\beta$ -estradiol ( $E2\beta$ ),  $17\alpha$ -  
54 ethynylestradiol (EE2), zearalenone,  $17\beta$ -testosterone, progesterone and L-leucine were  
55 purchased from Sigma, equol from Apin Chemicals (Abingdon, U.K.), diethylstilbestrol  
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3 (DES) from ICN. Isolute NH<sub>2</sub> extraction columns (100 mg) from IST (Hengoed, U.K.) and  
4  
5 Bond Elut C18 solid phase extraction columns (500 mg) from Varian (Harbor City, CA,  
6  
7 USA). Dextrose and yeast nitrogen base without amino acids and without ammonium  
8  
9 sulphate were obtained from Difco (Detroit, MI, USA). The minimal medium with L-leucine  
10  
11 medium (MM/L) consisted of yeast nitrogen base without amino acids and without  
12  
13 ammonium sulphate (1.7 g per l), dextrose (20 g per l) and ammonium sulphate (5 g per  
14  
15 l) and was supplemented with L-leucine (60 mg per l).

## 16 17 18 19 2.2 Samples and sample treatment

20  
21 As representatives for 20 animal feed samples, 7 wet pulp feed samples which are  
22  
23 normally used to feed pigs, 7 dry grinded (1 mm) feed samples which are normally used  
24  
25 to feed ruminants, poultry and pigs and 6 regular milk replacers which are normally used  
26  
27 to feed calves were used. The extraction procedure described below is partly comparable  
28  
29 to the procedure that is commonly applied to the isolation of steroids from urine samples  
30  
31 (Blankvoort et al., 2003; Nielen et al., 2004). Stock solutions of E2 $\beta$ , EE2, DES,  
32  
33 zearalenone, equol, testosterone and progesterone were prepared in DMSO (respectively  
34  
35 0.5; 0.5; 0.5; 100, 10000, 50 and 50  $\mu\text{g ml}^{-1}$ ). Feed samples were spiked with E2 $\beta$ , EE2  
36  
37 and DES at 5 and 10 ng per g, zearalenone at 1250 ng per g and equol at 200  $\mu\text{g per g}$ .  
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39 For extraction, 1 g of blank feed and spiked feed samples were mixed with 4 ml methanol  
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41 and 4 ml sodium acetate pH 4.8. Samples were incubated for 10 min in an ultrasonic bath  
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43 and subsequently mixed for 15 min head over head. Samples were centrifuged at 3500 g  
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45 and 4 ml of the upper liquid phase was brought in a glass tube. Next, the pH was  
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47 adjusted to 4.8 using 4 N acetic acid and the extract was subjected to solid phase  
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49 extraction (SPE) on a C18 column. This column was previously conditioned with 2.5 ml  
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51 methanol and after that with 2.5 ml sodium acetate pH 4.8. Subsequently, this column  
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53 was washed with 1.5 ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5 ml  
54  
55 sodium acetate pH 4.8, 3.0 ml water and finally with 2 ml methanol/water (50/50 v/v). The  
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57 column was air-dried and eluted with 4 ml acetonitrile. The eluate was applied to an NH<sub>2</sub>-  
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59 column that was previously conditioned with 3.0 ml acetonitrile. The acetonitrile eluate  
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thus obtained was evaporated to 3 ml under a stream of nitrogen gas. A 100  $\mu\text{l}$  or 200  $\mu\text{l}$

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3 aliquot of this extract, equivalent to respectively 0.017 or 0.033 g animal feed, was  
4 transferred to a 96 well plate in triplicate and 50  $\mu$ l of a 4% DMSO solution was added to  
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6 each well. To remove the acetonitrile, the plate was dried overnight in a fume cupboard  
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8 and was then ready to be screened on estrogenic activities with the yeast estrogen  
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10 bioassay. In the same way and in each series a reagent blank was prepared.  
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### 13 14 15 2.3 Yeast estrogen bioassay

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17 The yeast cytosensor expressing the human estrogen receptor  $\alpha$  (hER $\alpha$ ) and yeast  
18 enhanced green fluorescent protein (yEGFP), the latter in response to estrogens was  
19 developed in-house and has been described previously (Bovee et al., 2004a., 2004b). In  
20 short, an agar plate containing the selective MM/L medium was inoculated with the yeast  
21 ER $\alpha$  cytosensor from a frozen -80°C stock (20% glycerol v/v). The plate was incubated at  
22 30°C for 24-48 h and then stored at 4°C. The day before running the assay, a single  
23 colony of the yeast cytosensor was used to inoculate 10 ml of selective MM/L medium.  
24 This culture was grown overnight at 30°C with vigorous orbital shaking at 225 rpm. At the  
25 late log phase, the yeast ER $\alpha$  cytosensor was diluted in MM/L, giving an OD at 604 nm in  
26 the range of 0.07-0.13. For exposure in 96 well plates, aliquots of 200  $\mu$ l of this diluted  
27 yeast culture were pipetted into each well, already containing the extracts of the feed  
28 samples (see section 2.2). A 17 $\beta$ -estradiol dose-response curve was included in each  
29 exposure experiment. Aliquots of 200  $\mu$ l of the diluted yeast culture were pipetted into  
30 each well of a 96 well plate and exposure to different doses of 17 $\beta$ -estradiol was  
31 performed through the addition of 2  $\mu$ l of 17 $\beta$ -estradiol stock solutions in DMSO. Each  
32 feed sample extract and each 17 $\beta$ -estradiol stock was assayed in triplicate. Exposure  
33 was performed for 0 h and 24 h. Fluorescence at these time intervals was measured  
34 directly in a CytoFluor Multi-Well Plate Reader (Series 4000, PerSeptive Biosystems)  
35 using excitation at 485 nm and measuring emission at 530 nm. The densities of the yeast  
36 culture at these time intervals were also determined by measuring the OD at 630 nm.  
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38 This was done to check whether a feed sample was toxic for yeast.  
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### 2.4 Assay validation and data analysis

### *Detection capability $CC\beta$*

Extracts of 20 blank animal feed and 20 spiked animal feed samples (E2 $\beta$ , EE2 and DES at 10 ng g<sup>-1</sup>, zearalenone at 1.25  $\mu$ g g<sup>-1</sup> and equol at 200  $\mu$ g g<sup>-1</sup>) were analysed in the bioassay in order to determine the decision limit  $CC\alpha$  and the detection capability  $CC\beta$  of the yeast estrogen bioassay. In each series an extract of a reagent blank was made as well and was also analysed in the bioassay. Each sample extract was assayed in triplicate. Fluorescence signals of the 20 blank feed samples and the 20 spiked feed samples obtained after 24 h of exposure were corrected for the signals obtained at 0 h ( $t_{24}-t_0$ ) and were also corrected for the signal ( $t_{24}-t_0$ ) obtained with a reagent blank. All these signals are the mean of a triplicate. The extracts of the blank feed and their corresponding spikes were prepared on six different days and the 100  $\mu$ l aliquots were analysed in the yeast estrogen bioassay in five separate exposures. The 200  $\mu$ l aliquots of these extracts were analysed in the yeast estrogen assay in one exposure. These six sample treatments and exposures were performed within a time period of 3 months. In another experiment, extracts of the 20 blank feed samples spiked with E2 $\beta$ , EE2 and DES at 5 ng g<sup>-1</sup> were prepared in two days and 200  $\mu$ l aliquots of these extracts were analysed in the yeast estrogen bioassay in one exposure. In the context of EC Decision 2002/657 we define the mean signal of 20 blank animal feed samples plus 3 times the corresponding standard deviation as the decision limit  $CC\alpha$  ( $\alpha=1\%$ ). Samples with a signal below this  $CC\alpha$  are classified as compliant and samples with a signal above this  $CC\alpha$  are classified as suspect. The criterion for the decision limit  $CC\alpha$  is that all 20 blank feed samples give a signal below the determined decision limit  $CC\alpha$  and are thus classified as compliant ( $\alpha=1\%$ ). The criterion for the detection capability  $CC\beta$  is that at least 19 out of the 20 spiked samples have to give a signal above this  $CC\alpha$  and are thus classified as suspect ( $\beta=5\%$ ).

### *Specificity*

To determine the specificity of the yeast estrogen bioassay for screening estrogenic activity in animal feed, three blank feed samples, one wet, one dry and one milk replacer,

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3 were spiked with a high dose of  $17\beta$ -testosterone or progesterone ( $1000 \text{ ng g}^{-1}$ ) and 200  
4  $\mu\text{l}$  extracts were analysed in the bioassay. To check for interference, these three blank  
5  
6  $\mu\text{l}$  extracts were analysed in the bioassay. To check for interference, these three blank  
7  
8 feed samples were spiked with the high dose of either  $17\beta$ -testosterone or progesterone  
9  
10 in combination with a low dose of estrogens:  $\text{E}2\beta$ ,  $\text{EE}2$  and  $\text{DES}$  at  $10 \text{ ng g}^{-1}$ ,  
11  
12 zearalenone at  $1.25 \mu\text{g g}^{-1}$  and equol at  $200 \mu\text{g g}^{-1}$ .  
13

### 14 15 *Stability*

16  
17 For the determination of the stability of the feed samples, aliquots of 1 g of a blank and  
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19 spiked wet feed sample were stored at  $-20^\circ\text{C}$  and aliquots of 1 g of a blank and spiked  
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21 dry feed sample and of a blank and spiked milk replacer were stored at room temperature  
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23 in the dark. At certain times, 1 g aliquots of these three blank and spiked feed samples:  
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25  $\text{E}2\beta$  or  $\text{EE}2$  at  $5 \text{ ng g}^{-1}$ ,  $\text{DES}$  at  $10 \text{ ng g}^{-1}$ , zearalenone at  $1.25 \mu\text{g g}^{-1}$  and equol at  $200 \mu\text{g}$   
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27  $\text{g}^{-1}$ , were taken and extracts were made and 200  $\mu\text{l}$  aliquots were analysed in the  
28  
29 bioassay.  
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### 35 **3. Results and discussion**

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38 The performance characteristics detection capability  $\text{CC}\beta$ , specificity and stability of the  
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40 yeast estrogen bioassay for the screening of estrogenic activity in animal feed were  
41  
42 determined in order to validate the bioassay and to test its possible use to screen low  
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44 levels of estrogens in animal feed. There is no permitted limit for estrogens, but for the  
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46 routine screening an action level of 5 ng of  $17\beta$ -estradiol per gram feed was selected for  
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48 validation. This is very low, as with illegal use or accidents with pharmaceuticals, much  
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50 higher contents are expected. Theoretically, assuming no recovery loss during the sample  
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52 treatment, the 200  $\mu\text{l}$  extract, equivalent to 0.033 g of feed, of the  $5 \text{ ng g}^{-1}$   $17\beta$ -estradiol  
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54 spike that is added to a well in a final well volume of about 250  $\mu\text{l}$ , results in a final  
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56 concentration of 2.45 nM  $17\beta$ -estradiol in the well. We have shown previously (Bovee et  
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58 al., 2004b) that in the yeast bioassay the concentration where half-maximal activation is  
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60 reached ( $\text{EC}_{50}$ ), is about 0.7 nM for  $17\beta$ -estradiol and so theoretically the  $5 \text{ ng g}^{-1}$  spike

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3 of 17 $\beta$ -estradiol should easily be detected. Bovee et al. 2004b showed that the relative  
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5 estrogenic potency (REP) of a compound, defined as the ratio between the EC<sub>50</sub> of 17 $\beta$ -  
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7 estradiol and the EC<sub>50</sub> of that compound, is 1.2, 1, 0.005 and 0.0005 for EE2, DES,  
8  
9 zearalenone and equol respectively. This means that both EE2 and DES are about as  
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11 potent as E2 $\beta$  and that zearalenone and equol are respectively 200 and 2000 times less  
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13 potent than E2 $\beta$ . Therefore, both EE2 and DES were spiked at the same level as E2 $\beta$  (5  
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15 ng g<sup>-1</sup>) and zearalenone and equol were spiked at higher levels, respectively 1.25 and  
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17 200  $\mu$ g g<sup>-1</sup>. With the used clean-up procedure it is expected that the more apolar  
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19 compounds will have slightly lower recoveries. DES, the most apolar compound tested,  
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21 was therefore also spiked at the two times higher level of 10 ng g<sup>-1</sup>.

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23 In the routine screening of animal feed samples, the 200  $\mu$ l extracts were occasionally  
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25 (<1% of the samples) cytotoxic. In order to be able to screen these extracts on estrogenic  
26  
27 activity, 100  $\mu$ l instead of 200  $\mu$ l aliquots of these extracts were analysed in the bioassay.  
28  
29 The 100  $\mu$ l aliquots never showed cytotoxicity. In a second validation experiment, feed  
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31 samples were spiked with E2 $\beta$ , EE2 and DES at 10 ng g<sup>-1</sup>, zearalenone at 1.25  $\mu$ g g<sup>-1</sup> and  
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33 equol at 200  $\mu$ g g<sup>-1</sup> and 100  $\mu$ l aliquots of the extracts, equivalent to 0.017 g feed, were  
34  
35 analysed in the bioassay.

### 39 3.1 Detection capability (CC $\beta$ ) with 200 $\mu$ l aliquots of the feed sample extracts

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41 In this experiment feed samples were spiked with E2 $\beta$  and EE2 at 5 ng g<sup>-1</sup>, DES at 5 and  
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43 10 ng g<sup>-1</sup>, zearalenone at 1.25  $\mu$ g g<sup>-1</sup> and equol at 200  $\mu$ g g<sup>-1</sup>. Table I shows the results of  
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45 the yeast estrogen bioassay of the 20 blank and the 20 spiked feed samples using 200  $\mu$ l  
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47 of the extracts. The signals are the responses obtained after 24 h of exposure that are  
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49 corrected for the responses obtained at 0 h (t<sub>24</sub>-t<sub>0</sub>) and for the response (t<sub>24</sub>-t<sub>0</sub>) obtained  
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51 with a reagent blank. All these responses are the mean of a triplicate and in general the  
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53 %CV of these triplicates is less than 5% (data not shown). After 24 h of exposure there  
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55 were no differences in the OD at 630 nm, meaning that no toxic effects on the yeast could  
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57 be observed (data not shown). Most blank feed samples have corrected responses that  
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59 have a negative value (see Table I). These negative values are due to the relatively low  
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responses of these blank feed samples when compared to the corresponding reagent

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3 blank, for which they are corrected. Also the  $17\beta$ -estradiol standard curve corrected for  
4 the response obtained with a reagent blank starts with low negative values (see Figure 1).  
5  
6 Furthermore, these negative values are very low when compared to the signals that were  
7  
8 measured. For example, the  $t_0$  and  $t_{24}$  measurements of sample #1 were respectively  
9  
10  $101 \pm 3$  (standard deviation) and  $307 \pm 2$ , resulting in a  $t_{24}-t_0$  signal that was  $206 \pm 2$ .  
11  
12 The corresponding reagent blank gave a signal of  $222 \pm 4$  and as a result the corrected  
13  
14 signal for blank feed #1 is -16. However, the signals of the blank dry feed samples are the  
15  
16 lowest and although there is no toxic effect, these extracts probably contain substances  
17  
18 that give a little inhibitory effect in the bioassay. The mean response value of the 20 blank  
19  
20 feed samples  $X_B$  is -28 with a corresponding standard deviation  $S_B$  of 35. The calculated  
21  
22 decision limit  $CC\alpha$ , being the mean plus 3 times the standard deviation, for the corrected  
23  
24 fluorescence response is therefore 77. The use of the assay can be seen as a qualitative  
25  
26 ON/OFF method. Samples giving a signal lower than the  $CC\alpha$  are compliant: OFF.  
27  
28 Samples giving a response higher than the determined  $CC\alpha$  are suspect: ON. The results  
29  
30 in Table I demonstrate that the blank feed samples fulfil the  $CC\alpha$  criterion, meaning that  
31  
32 all blank feed samples give a response that is lower than the  $CC\alpha$ . All blank samples are  
33  
34 thus compliant (method result: OFF). For DES spiked at 5 ng per gram feed, 10 of the 20  
35  
36 spiked samples gave a response that is lower than the determined  $CC\alpha$  of 77. This  
37  
38 compound does not fulfil the  $CC\beta$  criterion at the 5 ng  $g^{-1}$  level. However, DES spiked at  
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40 10 ng  $g^{-1}$  fulfils the  $CC\beta$  criterion, as all 20 spiked samples gave a response that is higher  
41  
42 than the  $CC\alpha$  and are thus suspect (method result: ON). All but two of the  $E2\beta$ , EE2,  
43  
44 zearalenone and equol spikes gave a response that is higher than the determined  $CC\alpha$   
45  
46 and are thus classified as suspect samples. Only sample #4 for  $E2\beta$  and sample #4 for  
47  
48 EE2 showed a response that is lower than the  $CC\alpha$  and are thus classified as compliant.  
49  
50 These are false compliant results. However, overall the results fulfil the  $CC\beta$  criterion,  
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52 meaning that at least 19 out of the 20 spiked samples gave a signal above the  $CC\alpha$  and  
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54 are thus classified as suspect (method result: ON) ( $\beta=5\%$ ).  
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[INSERT TABLE I ABOUT HERE]

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3 In general the spikes in milk replacer gave about the same mean signals (X) as all 20  
4 feed samples, spikes in dry feed gave lower responses and spikes in wet feed showed  
5 higher responses. Probably the recovery of the spiked compounds is best from wet feed  
6 and recoveries from milk replacer and dry feed are lower. Recovery losses with the  
7 applied extraction procedure are mainly determined by the relative polarity of a  
8 compound and the interaction with matrix particles. So, the lower recoveries from milk  
9 replacer and dry feed could be expected as these matrices contain rather apolar particles,  
10 just as the compounds tested, and so interactions from the compounds with matrix  
11 particles are probably stronger for milk replacer and dry feed and are the strongest for  
12 DES, being the most apolar compound of the compounds tested. These recovery losses  
13 and/or the possible inhibitory effect in the bioassay of the dry feed extracts are probably  
14 the reason for the lower responses found with especially the DES spikes in dry feed.  
15 However, the bioassay method is not affected to such an extent that it does disturb the  
16 qualitative compliant/suspect (OFF/ON) screening principle of the method.  
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33 [INSERT FIGURE 1 ABOUT HERE]  
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### 37 3.2 Detection capability ( $CC\beta$ ) with 100 $\mu$ l aliquots of the feed sample extracts

38 In this experiment feed samples were spiked with 10  $\text{ng g}^{-1}$  of E2 $\beta$ , EE2 and DES, 1.25  
39  $\mu\text{g g}^{-1}$  of zearalenone or 200  $\mu\text{g g}^{-1}$  of equol. Table II shows the results of the yeast  
40 estrogen bioassay of the 20 blank and the 20 spiked feed samples using 100  $\mu$ l of the  
41 extracts. All these responses are the mean of a triplicate and in general the %CV of these  
42 triplicates was less than 5% (data not shown). Again, and as expected, there were no  
43 differences in the OD at 630 nm after 24 h of exposure, meaning that no toxic effects on  
44 the yeast were observed (data not shown). Just as for the 200  $\mu$ l aliquots, 100  $\mu$ l aliquots  
45 of the extracts of the blank feed samples have corrected responses with a negative value  
46 (see Table II). The mean response value of the 20 blank feed samples  $X_B$  was -11 with a  
47 corresponding standard deviation ( $S_B$ ) of 16. As a result the calculated decision limit  $CC\alpha$   
48 for the corrected fluorescence response was calculated to be 37. The results in Table II  
49 demonstrate that the blank feed samples fulfil the  $CC\alpha$  criterion, meaning that all blank  
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3 feed samples gave a response that was lower than the  $CC\alpha$ . As the rate of false suspect  
4 results is 1% ( $\alpha=1\%$ ), it is not expected to find a false suspect result. As can be seen in  
5 Table II, all but two of the E2 $\beta$ , EE2, DES, zearalenone and equol spikes gave a  
6 response that was higher than the determined  $CC\alpha$  and were thus classified as suspect  
7 samples. Only sample #2 for DES and sample #19 for equol gave a response that was  
8 lower than the  $CC\alpha$  and were thus classified as compliant. These are false compliant  
9 results. Sample #2 for DES is a real false compliant, but in sample #19 the equol was  
10 possibly not spiked. However, all spikes fulfil the  $CC\beta$  criterion, meaning that at least 19  
11 out of the 20 spiked samples gave a signal above the  $CC\alpha$  and are thus classified as  
12 suspect ( $\beta=5\%$ ).  
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25 [INSERT TABLE II ABOUT HERE]  
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29 Although the validation was performed as a qualitative screening method for the  
30 determination of estrogenic activity in animal feed and is used as an ON/OFF assay, the  
31 responses of the feed extracts can be converted to concentrations using a 17 $\beta$ -estradiol  
32 standard dose-response curve that is also corrected for a reagent blank (see Figure 1).  
33 When the  $CC\alpha$  of 37 is calculated from this standard curve this quantitative approach  
34 results in a concentration of 0.23 nM 17 $\beta$ -estradiol equivalents in the well. This  
35 corresponds to 0.94 ng E2 $\beta$  equivalents per gram of feed. In the same way the mean  
36 response values of the spiked samples can be converted to concentrations and contents  
37 of E2 $\beta$  equivalents. The 17 $\beta$ -estradiol, EE2 and DES spikes are made at a level of 10 ng  
38 per gram and as the mean response of 575 of the 17 $\beta$ -estradiol spikes corresponds to  
39 4.4 ng E2 $\beta$  equivalents per gram, this means that the recovery of the method for 17 $\beta$ -  
40 estradiol was 44%. For EE2, which has a relative estrogenic potency (REP) of 1.2, 100%  
41 recovery would result in 12 ng E2 $\beta$  equivalents per gram and so the calculated content of  
42 5.1 ng E2 $\beta$  equivalents per gram means that the recovery for this compound was 42%.  
43 For DES, which has a REP of 1, a content of 2.3 ng E2 $\beta$  equivalents per gram was  
44 calculated and thus the calculated recovery for this compound was 23%. Zearalenone  
45 has a REP of 4.6E-3 and was spiked at a level of 1250 ng per gram, thus 100% recovery  
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3 would result in 5.8 ng E2 $\beta$  equivalents per gram. A content of 2.6 ng E2 $\beta$  equivalents per  
4 gram was calculated and thus the recovery of the method for this compound was 45%.  
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6  
7 The recovery for equol could not be calculated as this compound was spiked with a  
8 higher amount, resulting in a mean response value that was equal to the maximum  
9 response of the 17 $\beta$ -estradiol standard dose-response curve. Although this quantitative  
10 approach is beyond the scope of this paper and is not needed for the validation of a  
11 qualitative screening method, it shows that the recovery of the sample treatment is  
12 about 45% for 17 $\beta$ -estradiol, 17 $\alpha$ -ethynylestradiol and zearalenone. For DES, the most  
13 apolar compound tested, the recovery is about 25%.  
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### 23 3.3 Specificity and interference

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25 The specificity of the yeast estrogen bioassay was determined with three blank animal  
26 feed samples, one wet, one dry and one milk replacer that were spiked with a high dose  
27 of 17 $\beta$ -testosterone or progesterone (1000 ng g<sup>-1</sup>). Interference was checked with these  
28 three blank animal feed samples by spiking them with a low dose of E2 $\beta$ , EE2, DES at ng  
29 g<sup>-1</sup>, zearalenone at 1.25  $\mu$ g g<sup>-1</sup> and equol at 200  $\mu$ g g<sup>-1</sup> in combination with the high dose  
30 of either 17 $\beta$ -testosterone or progesterone. Extracts were made and 200  $\mu$ l aliquots were  
31 analysed in the bioassay. Fluorescence signals at 24 h were corrected for the signals  
32 obtained at 0 h and the response of a reagent blank. Results are shown in Table III.  
33 Strictly seen, the milk replacer spiked with 1000 ng progesterone per gram gave a false  
34 suspect result. However, this milk replacer spiked with 1000 ng progesterone per gram  
35 gave a signal of 80 that was just above the determined decision limit CC $\alpha$  of 77. When  
36 looking at the data in Table III, they show that neither the androgen 17 $\beta$ -testosterone nor  
37 the gestagen progesterone gave a response in the bioassay, as the blank samples gave  
38 about the same response as the corresponding 17 $\beta$ -testosterone and progesterone  
39 spiked samples. Results in Table III also show that both of these compounds do not  
40 interfere with the screening result of the estrogenic compounds E2 $\beta$ , DES, EE2,  
41 zearalenone or equol, as all signals of the estrogen spiked samples are well above the  
42 CC $\alpha$  value of 77. Moreover, responses of the estrogen spiked samples are about the  
43 same as spiking them in combination with the high dose of either 17 $\beta$ -testosterone or  
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3 progesterone. Despite the one false suspect result, the data demonstrate the specificity of  
4  
5 the bioassay.  
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9 [INSERT TABLE III ABOUT HERE]  
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### 11 12 13 3.4 Stability of estrogens in feed

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15 The stability of the estrogenic compounds in animal feed samples was determined with  
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17 three animal feed samples, one wet, one dry and one milk replacer that were spiked.  
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19 Aliquots of 1 g of a blank and spiked wet feed sample were stored at -20°C and aliquots  
20  
21 of 1 g of a blank and spiked dry feed sample and of a blank and spiked milk replacer  
22  
23 were stored at room temperature in the dark. At certain times, 1 g aliquots of these three  
24  
25 blank and spiked feed samples: E2 $\beta$  or EE2 at 5 ng g<sup>-1</sup>, DES at 10 ng g<sup>-1</sup>, zearalenone at  
26  
27 1.25  $\mu$ g g<sup>-1</sup> and equol at 200  $\mu$ g g<sup>-1</sup>, were taken and extracts were made and 200  $\mu$ l  
28  
29 aliquots were analysed in the bioassay. Fluorescence signals at 24 h were corrected for  
30  
31 the signals obtained at 0 h and the response of a reagent blank. Results are shown in  
32  
33 Table IV. Only the equol spike in the wet feed that was stored at room temperature in the  
34  
35 dark for 55 days gave a corrected response of -15 that was below that of the CC $\alpha$  value  
36  
37 of 77. This is a false compliant. The corresponding blank wet feed gave a response of  
38  
39 379 and is a false suspect. However, it is much more likely that there was an interchange  
40  
41 of these two samples. The 200  $\mu$ l extracts of the dry feed at day 0 were toxic to the yeast  
42  
43 cells. This could be seen at the OD at 630 nm, while the OD was about 1.0 for the  
44  
45 controls, the wet feed and the milk replacer, the OD at 630 nm with 200  $\mu$ l extracts of this  
46  
47 dry feed was below 0.6 (data not shown). Extracts are considered to be toxic if the OD  
48  
49 measured at 630 nm is less than 70% of the OD of the control samples. Extracts of day 0  
50  
51 were kept at 4°C and from day 2 on, exposures with the extracts of this dry feed were  
52  
53 performed with 100  $\mu$ l instead of 200  $\mu$ l. As a consequence, a CC $\alpha$  value of 37 instead of  
54  
55 77 was applied. The 100  $\mu$ l aliquots of this dry feed sample were analysed and were  
56  
57 never cytotoxic. The 200  $\mu$ l aliquots of this dry feed sample were not always cytotoxic and  
58  
59 when not cytotoxic they fulfilled the criteria (data not shown). The data in Table IV  
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demonstrate that the feed samples can be stored at their specific conditions for up to 98

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3 days, without disrupting the screening result, assuming that the blank and equol spike of  
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5 the wet feed were interchanged at day 55.  
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9 [INSERT TABLE IV ABOUT HERE]  
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13 3.5 Application of the yeast estrogen bioassay for the screening of estrogenic activity in  
14 animal feed

15  
16 This method is in routine use at RIKILT for more than one year now. In that period, the  
17 method was applied more than 40 times to series of about 20 feed samples and showed  
18 good robustness: feed extracts were almost never cytotoxic (less than 1% of the  
19 samples) and when cytotoxic, the 100  $\mu$ l method could be used. Reagent blanks were  
20 always found compliant and the spiked reagent blanks were always screened suspect.  
21 Figure 2 shows a control chart, showing the decision limit  $CC\alpha$  and the corrected  
22 responses for the reagent blank, blank feed control, spiked reagent blank and the spiked  
23 feed control. Only once a blank feed control was found suspect and once a spiked feed  
24 control was found compliant. However, the extract of this spiked feed control was  
25 coloured. As this control feed was used several times, showing no colour and when  
26 spiked was always found suspect, this indicates that only once the clean-up procedure  
27 did not work optimally, resulting in a coloured extract that gave a signal just below the  
28 determined  $CC\alpha$ . The one time that a blank feed control was found suspect, the signal  
29 was just above the determined  $CC\alpha$ . Just as for the stability study, the data in Figure 2  
30 demonstrate that the method is robust and that the outcome of the assay,  
31 compliant/suspect, is reproducible.  
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50 [INSERT FIGURE 2 ABOUT HERE]  
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54 The method is validated as a qualitative screening method for estrogenic activity in feed  
55 and thus suspect screened samples need to be confirmed. Therefore, we have set up a  
56 liquid chromatography (LC) system that fractionates feed extracts using a dual 96 well  
57 fraction collector system via effluent splitting. The LC-fractions in the first 96 well plate are  
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3 used for the detection of estrogenic activity using the yeast estrogen bioassay. The  
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5 suspect well numbers are then correlated with the LC retention time and results from the  
6  
7 analysis of the corresponding "suspect" wells in the second 96 well plate with  
8  
9 QTOFMS/MS with exact mass measurement. Figure 3 shows this generic setup for the  
10  
11 fractionation and identification of (un)known bioactive substances using  
12  
13 LC/bioassay/QTOFMS(/MS) (Nielen et al. 2004; Nielen et al., in press).  
14

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17 [INSERT FIGURE 3 ABOUT HERE]  
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21 During the MPA-incident in the European Union in 2002 there were problems with  
22  
23 pregnant pigs in breeding farms (Van Leengoed et al., 2002). To determine the cause of  
24  
25 the problems, wet feed samples were sent to RIKILT for testing. Part of this testing was  
26  
27 to investigate these samples with our estrogen bioassay. The samples were screened  
28  
29 suspect in the estrogen bioassay. Knowing that the estrogen bioassay is specific for  
30  
31 estrogens, based on the fact that other hormones like  $17\beta$ -testosterone, progesterone  
32  
33 and medroxyprogesterone 17-acetate (MPA) do not give a response in this test (Bovee et  
34  
35 al., 2004b), the responsible substances had to be estrogenic. It was decided to  
36  
37 fractionate an extract. Figure 4 shows the estrogenicity biogram of a fractionated wet feed  
38  
39 and shows that fraction number 24 and 25 are estrogenic. In this system the  
40  
41 corresponding retention time of these fractions was equal to that of  $17\beta$ -estradiol. This  
42  
43 was the first indication that hormones could be responsible for the problems in the  
44  
45 pregnant pigs and that  $17\beta$ -estradiol might be involved. This was confirmed by GC/MS  
46  
47 analysis. The identified  $17\beta$ -estradiol turned out to be part of the problem, since early  
48  
49 thereafter it became clear that MPA was the main issue in the feed contamination. As  
50  
51 hormones used for hormone replacement therapy (HRT) and the female contraceptive  
52  
53 are two major pharmaceuticals, both containing large amounts of estrogens, this  
54  
55 demonstrates the applicability of the bioassay method as an early warning system for  
56  
57 pharmaceutical waste in animal feed. This is even true in cases where the estrogens are  
58  
59 part of the contamination, because the test is not influenced by the presence of other  
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3 hormones, i.e. there is no interference. It also demonstrates that the bioassay can be  
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5 used to identify a fraction that contains the estrogenic activity.  
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9 [INSERT FIGURE 4 ABOUT HERE]  
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11  
12 Figure 5 shows the estrogenicity biogram of a fractionated fish feed that was found  
13 suspect in our routine estrogen bioassay method. Figure 5 shows that fraction number 22  
14 and 23 are estrogenic. In this system (see Figure 3) the corresponding retention time  
15 from 7.00 to 7.66 minutes was equal to that of 17 $\beta$ -estradiol. Using the LC/bioassay/MS  
16 setup the 17 $\beta$ -estradiol was indeed confirmed by exact mass measurement in both  
17 fractions. This demonstrates the applicability of the bioassay method as an early warning  
18 system for hormone abuse. It also demonstrates that the bioassay can be used as an  
19 inexpensive LC detector to identify a fraction that contains the estrogenic activity. The  
20 natural steroids are orally not very active, but the 17-alkylated steroids, like the oral  
21 contraceptive 17 $\alpha$ -ethynylestradiol, are usually orally active in mammals and besides  
22 as waste, might thus be abused in animal feed. Less well known is the illegal use of  
23 hormones in aquaculture. In eel the natural estrogen 17 $\beta$ -estradiol is orally effective.  
24 Besides the growth effect, 17 $\beta$ -estradiol affects the sex determination of eels. After 1  
25 year, 70% of eels fed with 60 mg 17 $\beta$ -estradiol/kg in their diet were identified as female,  
26 compared to 26% of the control group (Degani and Kushnirov, 1992). In another study  
27 these percentages were 88% in the group treated with 17 $\beta$ -estradiol and 6% in the  
28 control group. This latter study also showed that after 600 days, males reached a weight  
29 of 114,1  $\pm$  4.3 and the females 171  $\pm$  11.7 (g  $\pm$  SE) (Degani et al., 2003). Thus the abuse  
30 of 17 $\beta$ -estradiol for the growth of eel can be very lucrative.  
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52 [INSERT FIGURE 5 ABOUT HERE]  
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## Conclusions

The data presented and the determined performance characteristics prove that the yeast estrogen bioassay can detect low levels of  $17\beta$ -estradiol ( $5 \text{ ng g}^{-1}$ ),  $17\alpha$ -ethynylestradiol ( $5 \text{ ng g}^{-1}$ ), diethylstilbestrol ( $10 \text{ ng g}^{-1}$ ), zearalenone ( $1.25 \text{ } \mu\text{g g}^{-1}$ ) and equol ( $200 \text{ } \mu\text{g g}^{-1}$ ) in animal feed by using  $200 \text{ } \mu\text{l}$  of the extracts. All 20 blank feed samples gave a response lower than the determined  $\text{CC}\alpha$  of 77 and thus there were no false suspect results. Signals of all 20 DES, zearalenone and equol spikes were higher than the determined  $\text{CC}\alpha$  of 77 and thus fulfilled the  $\text{CC}\beta$  criterion. Signals of 19 out of 20  $17\beta$ -estradiol and  $17\alpha$ -ethynylestradiol spikes were higher than the determined  $\text{CC}\alpha$  of 77 and thus also fulfilled the  $\text{CC}\beta$  criterion. When samples are cytotoxic,  $100 \text{ } \mu\text{l}$  of the extracts instead of  $200 \text{ } \mu\text{l}$  can be used. As a consequence, the applied  $\text{CC}\alpha$  must be 37 instead of 77 and the action levels of  $17\beta$ -estradiol and  $17\alpha$ -ethynylestradiol change from 5 to 10 ng per gram of feed.

High levels of the androgen  $17\beta$ -testosterone or the gestagen progesterone did not give a response in the bioassay, nor did they interfere with the screening result of spiked estrogens at low levels. The data demonstrate that the bioassay is specific for estrogenic substances. The observation that feed samples could be stored at their specific conditions for up to 98 days without influencing the screening result showed that estrogens in feed are stable. The stability study also demonstrates that the outcome of the assay, compliant/suspect, is reproducible and that the procedure is robust. As all the performance characteristics met the criteria that were put forward in EC Decision 2002/657 for validation of a qualitative screening method, the above described clean-up/yeast estrogen bioassay procedure is proven to be valid for the determination of estrogenic activity in animal feed. The clean-up procedure for feed samples is relatively simple and the yeast estrogen bioassay, using yEGFP as a reporter protein, is sensitive, rapid, convenient and reproducible. Combined this resulted in a low cost bioassay that is suited to be used as a high throughput system for the screening of estrogenic activity in complex animal feed samples. Due to the good sensitivity of the bioassay, only 1 gram of feed is enough to be processed. This results in a 3 ml acetonitrile extract of which 0.6 ml

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3 is needed for the bioassay screening in triplicate while the remaining 2.4 ml can be used  
4  
5 for identification and conformation using the LC/bioassay/QTOFMS setup.  
6

7 This method is in routine use at RIKILT for more than one year now. It showed good  
8  
9 robustness: feed extracts were almost never cytotoxic (less than 1% of the samples).  
10  
11 Reagent blank and blank feed controls were always found compliant and the spiked  
12  
13 reagent blank and spiked feed controls were always screened suspect. The examples of  
14  
15 the MPA-incident with wet pig feed and the fish feed demonstrate the applicability of the  
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17 bioassay method as an early warning system for pharmaceutical waste and hormone  
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19 abuse respectively.  
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25  
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28 Food Quality.  
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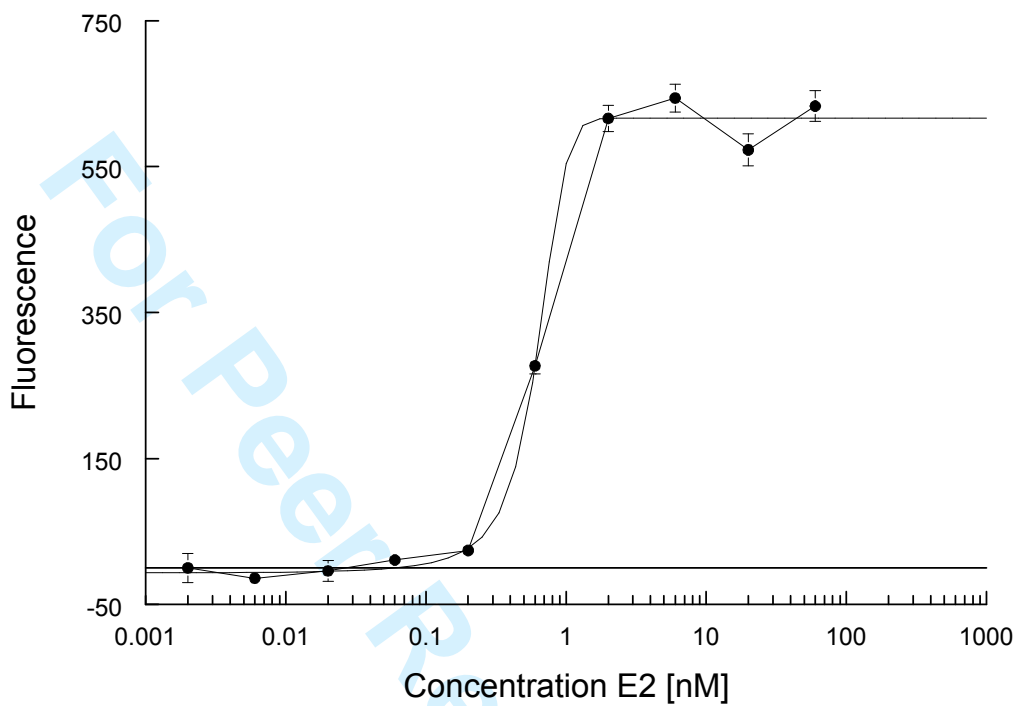


Figure 1.

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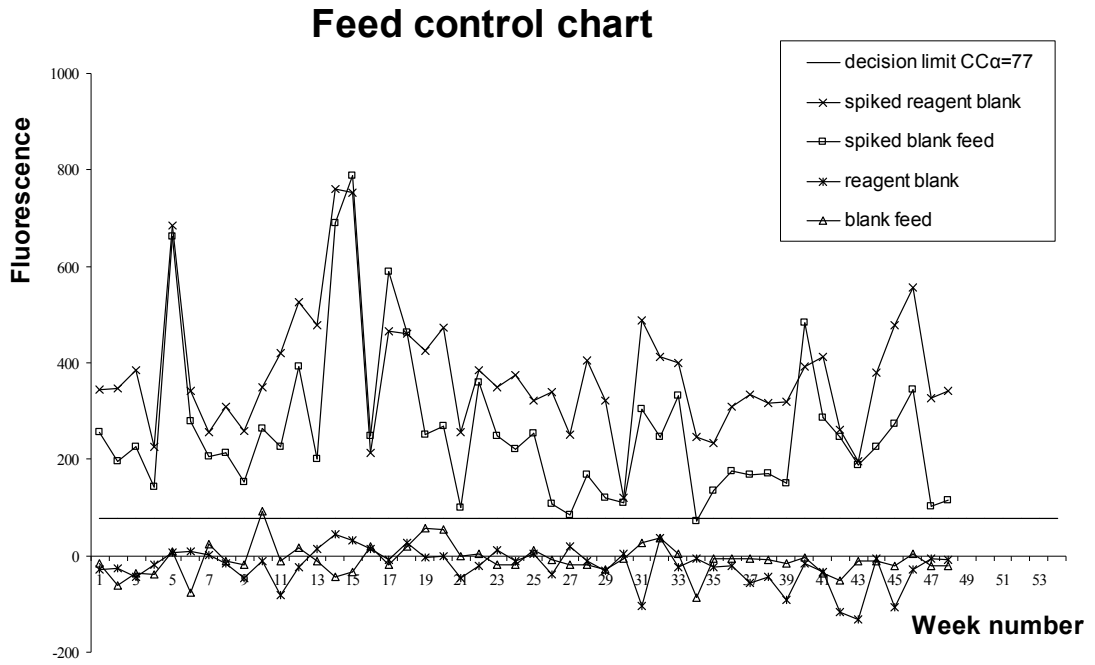


Figure 2.

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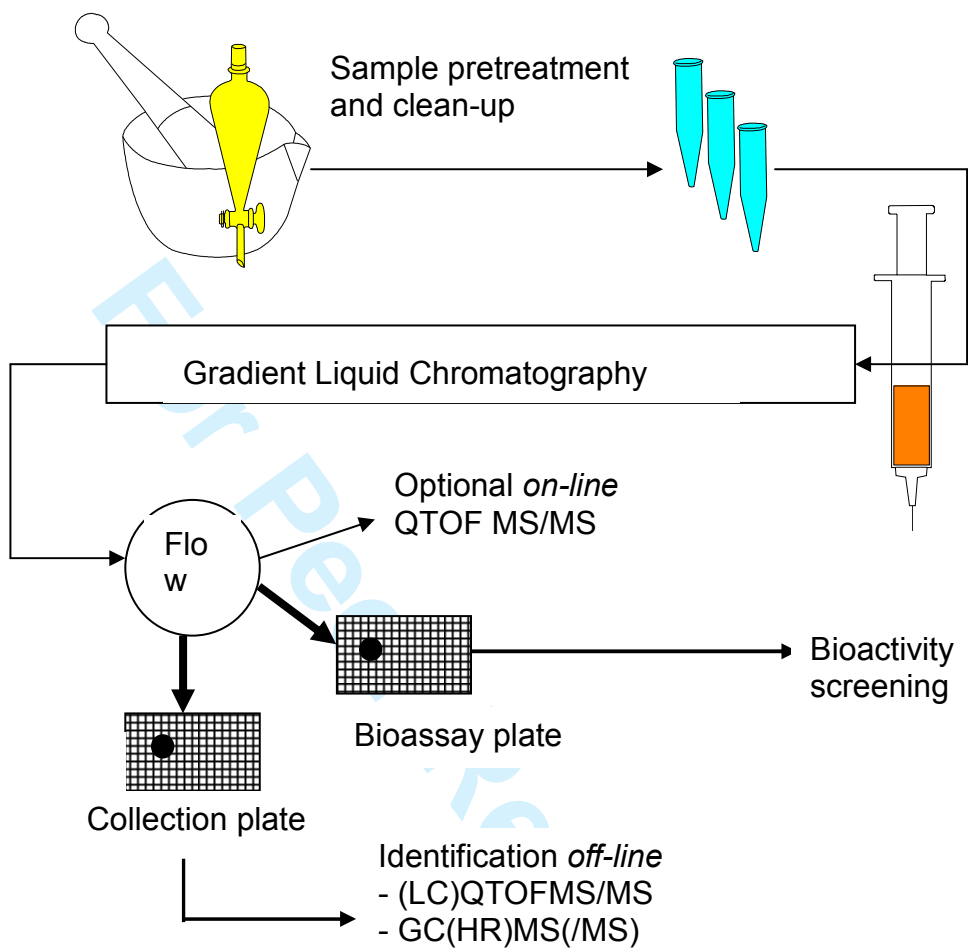


Figure 3.

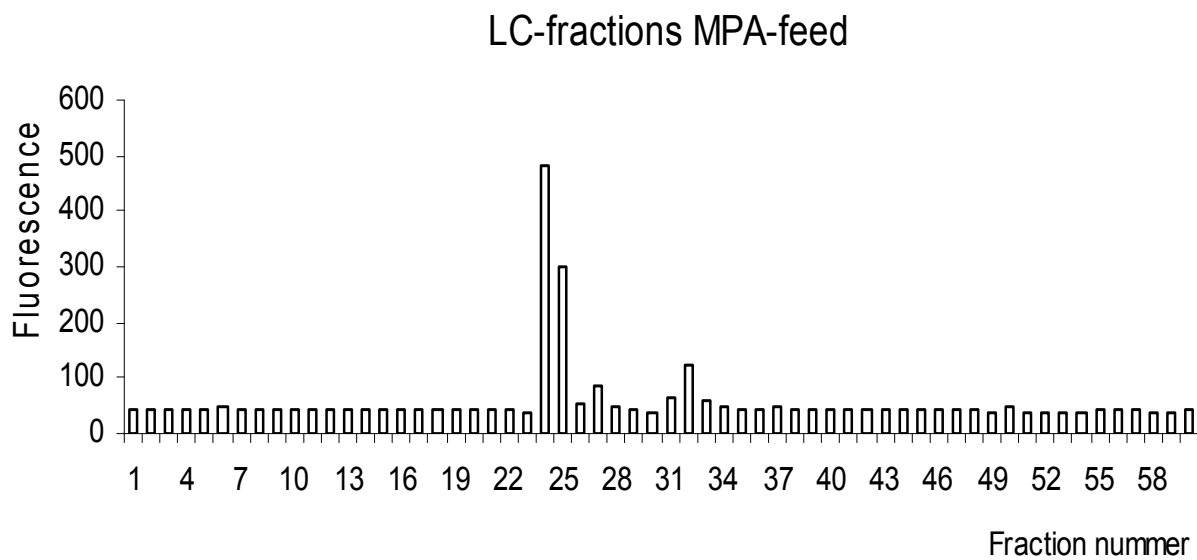


Figure 4.

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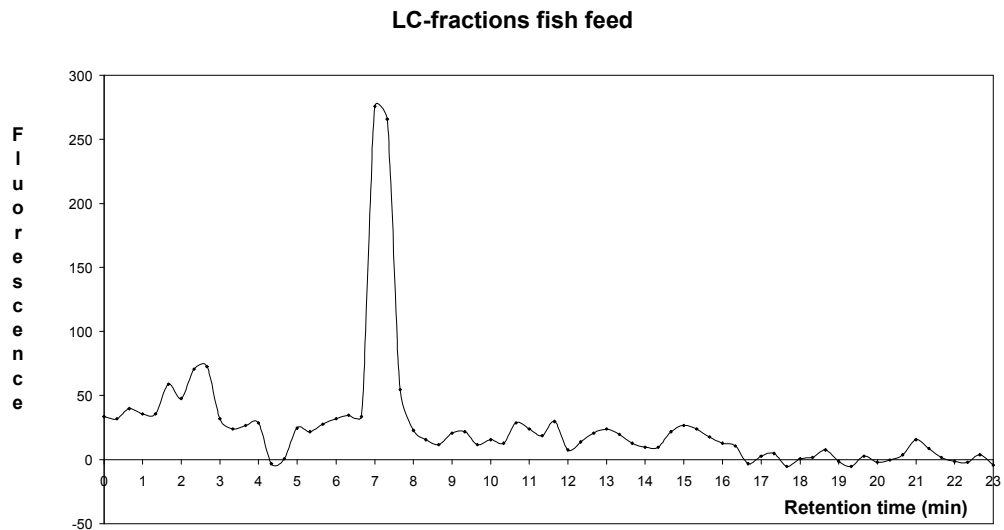


Figure 5.

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## Legends

Figure 1. Response of the yeast estrogen cytosensor after a 24 h exposure to 17 $\beta$ -estradiol. Exposure was started by adding 2  $\mu$ l of a 17 $\beta$ -estradiol stock solution in DMSO to 200  $\mu$ l of a yeast culture. Fluorescence was determined after 0 and 24 hours as described in Experimental (see section 2.3 Yeast estrogen bioassay). Fluorescence signals are the mean of a triplicate with sd and are corrected for the signals obtained at 0 h and a reagent blank.

Figure 2. Longterm response (n=48) in the yeast estrogen bioassay of the reagent blank, the reagent blank spiked with 5 ng 17 $\beta$ -estradiol per gram, the blank feed sample and a blank feed sample spiked with 5 ng 17 $\beta$ -estradiol per gram.

Figure 3. Generic setup for the fractionation and identification of unknown bioactive substances using LC/bioassay/QTOFMS(/MS).

Figure 4. Estrogenicity biogram of a wet feed sample from the MPA-incident that was found suspect in the yeast estrogen bioassay. Extracts were separated on a C18-column and fractions of 0.33 minutes were collected and tested in the bioassay.

Figure 5. Estrogenicity biogram of a fish feed sample that was suspect in the routine screening. Extracts were separated on a C18-column and fractions of 0.33 minutes were collected and tested in the bioassay.



Table I

Mean (X) fluorescence response in the yeast estrogen bioassay of 20 blank and 20 spiked feed samples and the determination of the decision limit  $CC\alpha$  using 200  $\mu$ l aliquots of the extracts for exposure.

Feed sample #	Mean (X) <sup>a</sup>	SD (S) <sup>a</sup>	CC $\alpha$ <sup>a</sup>	day 1										day 2									
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Blank feed	-28	35	77	milk	dry	milk	dry	milk	dry	milk	dry	dry	dry	dry	milk	milk	wet	wet	wet	wet	wet	wet	wet
E2 $\beta$ 5 ng g <sup>-1(b)</sup>	342	116		300	221	360	<b>64</b>	320	372	452	247	128	203	363	411	468	480	447	438	424	406	361	365
EE2 5 ng g <sup>-1(b)</sup>	314	117		299	155	312	<b>51</b>	297	307	466	167	97	237	363	382	357	446	417	398	427	411	328	356
DES 5 ng g <sup>-1(b)</sup>	129	123		87	<b>20</b>	<b>35</b>	<b>-72</b>	<b>36</b>	118	259	<b>52</b>	<b>-16</b>	<b>70</b>	<b>36</b>	<b>57</b>	129	290	255	291	278	310	<b>59</b>	292
DES 10 ng g <sup>-1(c)</sup>	315	181		264	169	853	133	194	270	507	154	180	179	648	139	285	370	301	355	323	401	252	324
zearalenone 1.25 $\mu$ g g <sup>-1(c)</sup>	285	101		300	214	304	118	268	253	556	190	160	206	490	247	259	332	343	338	297	297	239	290
equol 200 $\mu$ g g <sup>-1(c)</sup>	342	77		300	414	470	211	303	299	398	283	233	238	420	269	475	387	387	392	323	395	338	302

<sup>a</sup> Mean, SD and  $CC\alpha$  ( $CC\alpha$  = Mean of the blank + 3.0\*SD of the blank) are determined from 20 feed samples. In **bold and grey** the samples that not fulfil the criterion.

<sup>(b)</sup> The extracts were prepared on two different days and were analysed in the yeast estrogen bioassay in one exposure. These sample preparations and the exposure were performed within a time period of three weeks.

<sup>(c)</sup> The extracts were prepared on six different days (see Table 2) and the 200  $\mu$ l aliquots were analysed in the yeast estrogen bioassay in one exposure. These sample preparations and the exposure were performed within a time period of 3 months.

Table II

Mean (X) fluorescence response in the yeast estrogen bioassay of 20 blank and 20 spiked feed samples and the determination of the decision limit  $CC\alpha$  using 100  $\mu$ l aliquots of the extracts for exposure.

Feed sample #	Mean (X) <sup>a</sup>	SD (S) <sup>a</sup>	CC $\alpha$ <sup>a</sup>	day 1			day 2		day 3				day 4					day 5				day 6	
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Blank feed	-11	16	37	milk	dry	wet	milk	dry	milk	wet	wet	wet	dry	milk	dry	wet	wet	dry	dry	dry	wet	milk	milk
E2 $\beta$ 10 ng g <sup>-1</sup>	575	236		274	223	547	493	268	270	609	521	640	672	891	696	1046	899	249	529	596	849	590	644
EE2 10 ng g <sup>-1</sup>	600	233		332	205	632	635	245	355	635	565	700	726	910	668	1034	878	277	537	566	933	546	621
DES 10 ng g <sup>-1</sup>	239	164		68	<b>36</b>	208	129	80	71	341	292	335	303	418	148	558	421	96	193	118	587	116	263
zearalenone 1.25 $\mu$ g g <sup>-1</sup>	312	180		116	92	216	152	125	139	379	292	379	533	734	389	544	358	147	221	351	601	247	226
equol 200 $\mu$ g g <sup>-1</sup>	658	303		313	353	632	736	339	466	734	520	742	887	1101	920	1039	938	383	860	842	1011	<b>-15</b>	366

<sup>a</sup> Mean, SD and  $CC\alpha$  are determined from 20 feed samples. The extracts were prepared on six different days and were analysed in the yeast estrogen bioassay in five separate exposures. These sample preparations and exposures were performed within a time period of 3 months. In **bold and grey** the samples that not fulfil the criterion.

Table III

Specificity and interference of the yeast estrogen bioassay, checked with feed samples spiked with a high dose of 17 $\beta$ -testosterone (A) or progesterone (B).

Feed sample	dry feed			wet feed			milk replacer		
Addition <sup>a</sup>	none	A	B	none	A	B	none	A	B
Blank feed	-14	-4	-3	-61	-58	-61	58	68	<b>80</b>
E2 $\beta$ 10 ng g <sup>-1</sup>	328	331	335	373	370	349	1273	1271	1127
EE2 10 ng g <sup>-1</sup>	321	329	340	377	348	357	692	891	1148
DES 10 ng g <sup>-1</sup>	191	199	182	283	329	255	739	683	638
zearalenone 1.25 $\mu$ g g <sup>-1</sup>	181	175	200	241	201	193	400	363	334
equol 200 $\mu$ g g <sup>-1</sup>	338	382	341	342	378	357	1081	1209	1307

<sup>a</sup> The specificity of the yeast estrogen bioassay was determined with three blank feed samples that were spiked with a high dose of 17 $\beta$ -testosterone (A) or progesterone (B), both at a level of 1000 ng per gram. Interference was checked with the same three feed samples by spiking them with a low dose of E2 $\beta$ , EE2, DES, zearalenone or equol in combination with the high dose of either 17 $\beta$ -testosterone or progesterone. Exposures were performed with 200  $\mu$ l aliquots of the extracts. In **bold and grey** the samples that not fulfil the criterion.

Table IV

Stability of estrogens in animal feed.

Days at room temp. in the dark	0	0	2	7	14	28	55	98
Extract volume for exposure (µl)	200	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
<b>Dry feed</b> blank	tox	-76	-82	-70	-73	-79	-54	-74
E2β 5 ng g <sup>-1</sup>	tox	77	77	213	201	213	138	171
EE2 5 ng g <sup>-1</sup>	tox	51	80	193	198	232	141	173
DES 10 ng g <sup>-1</sup>	tox	79	81	148	165	213	109	111
zearalenone 1.25 µg g <sup>-1</sup>	tox	46	58	221	229	276	93	411
equol 200 µg g <sup>-1</sup>	tox	145	151	478	466	550	232	329
Days at room temp. in the dark		0	2	7	14	28	55	98
Extract volume for exposure (µl)		200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>
<b>Milk replacer</b> blank		-16	5	-8	-14	-18	-15	-13
E2β 5 ng g <sup>-1</sup>		258	335	257	626	629	333	574
EE2 5 ng g <sup>-1</sup>		242	286	547	627	635	360	590
DES 10 ng g <sup>-1</sup>		246	360	232	576	594	356	544
zearalenone 1.25 µg g <sup>-1</sup>		174	242	194	439	582	240	300
equol 200 µg g <sup>-1</sup>		199	317	320	607	755	331	444
Days at -20 °C		0	2	7	14	28	55	98
Extract volume for exposure (µl)		200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>
<b>Wet Feed</b> blank		-1	-1	28	-1	-8	<b>379</b>	-16
E2β 5 ng g <sup>-1</sup>		313	487	363	682	719	403	588
EE2 5 ng g <sup>-1</sup>		309	377	417	679	772	477	546
DES 10 ng g <sup>-1</sup>		309	455	433	658	748	477	586
zearalenone 1.25 µg g <sup>-1</sup>		261	304	329	528	744	434	381
equol 200 µg g <sup>-1</sup>		264	342	285	638	812	<b>-15</b>	430

<sup>a</sup> Exposures were performed with 100 µl aliquots of the extracts, because 200 µl of these extracts were toxic for the yeast cells. The corresponding CC<sub>α</sub> value is 37 (see Table 1).

<sup>b</sup> Exposures were performed with 200 µl aliquots of the extracts. The corresponding CC<sub>α</sub> value is 77 (see Table 2). In **bold and grey** the samples that not fulfil the criterion.

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Feed sample #	Mean (X) <sup>a</sup>	SD (S) <sup>a</sup>	CC <sub>d</sub> <sup>a</sup>	day 1										day 2									
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Blank feed	-28	35	77	milk	dry	milk	dry	milk	dry	milk	dry	dry	dry	dry	milk	milk	wet	wet	wet	wet	wet	wet	
E2β 5 ng g <sup>-1(b)</sup>	342	116		-16	9	-9	-101	-28	-43	9	-41	-89	-90	-87	-13	-14	-7	-15	-12	-4	-6	-2	-9
EE2 5 ng g <sup>-1(b)</sup>	314	117		300	221	360	<b>64</b>	320	372	452	247	128	203	363	411	468	480	447	438	424	406	361	365
DES 5 ng g <sup>-1(b)</sup>	129	123		299	155	312	<b>51</b>	297	307	466	167	97	237	363	382	357	446	417	398	427	411	328	356
DES 10 ng g <sup>-1(b)</sup>	129	123		87	<b>20</b>	<b>35</b>	<b>-72</b>	<b>36</b>	118	259	<b>52</b>	<b>-16</b>	<b>70</b>	<b>36</b>	<b>57</b>	129	290	255	291	278	310	<b>59</b>	292
DES 10 ng g <sup>-1(c)</sup>	315	181		264	169	853	133	194	270	507	154	180	179	648	139	285	370	301	355	323	401	252	324
zearalenone 1.25 µg g <sup>-1(c)</sup>	285	101		300	214	304	118	268	253	556	190	160	206	490	247	259	332	343	338	297	297	239	290
equol 200 µg g <sup>-1(c)</sup>	342	77		300	414	470	211	303	299	398	283	233	238	420	269	475	387	387	392	323	395	338	302

Feed sample #	Mean (X) <sup>a</sup>	SD (S) <sup>a</sup>	CC <sub>d</sub> <sup>a</sup>	day 1			day 2		day 3				day 4					day 5				day 6	
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Blank feed	-11	16	37	milk	dry	wet	milk	dry	milk	wet	wet	wet	dry	milk	dry	wet	wet	dry	dry	dry	wet	milk	milk
E2 $\beta$ 10 ng g <sup>-1</sup>	575	236		-11	12	-4	9	-32	-10	-4	-6	-34	-26	4	-31	-5	-6	-36	6	-37	1	-10	5
EE2 10 ng g <sup>-1</sup>	600	233		274	223	547	493	268	270	609	521	640	672	891	696	1046	899	249	529	596	849	590	644
DES 10 ng g <sup>-1</sup>	239	164		332	205	632	635	245	355	635	565	700	726	910	668	1034	878	277	537	566	933	546	621
zearalenone 1.25 $\mu$ g g <sup>-1</sup>	312	180		68	36	208	129	80	71	341	292	335	303	418	148	558	421	96	193	118	587	116	263
equol 200 $\mu$ g g <sup>-1</sup>	658	303		116	92	216	152	125	139	379	292	379	533	734	389	544	358	147	221	351	601	247	226
				313	353	632	736	339	466	734	520	742	887	1101	920	1039	938	383	860	842	1011	-15	366

Feed sample Addition <sup>a</sup>	dry feed			wet feed			milkreplacer		
	none	A	B	none	A	B	none	A	B
Blank feed	-14	-4	-3	-61	-58	-61	58	68	<b>80</b>
E2β 10 ng g <sup>-1</sup>	328	331	335	373	370	349	1273	1271	1127
EE2 10 ng g <sup>-1</sup>	321	329	340	377	348	357	692	891	1148
DES 10 ng g <sup>-1</sup>	191	199	182	283	329	255	739	683	638
zearalenone 1.25 μg g <sup>-1</sup>	181	175	200	241	201	193	400	363	334
equol 200 μg g <sup>-1</sup>	338	382	341	342	378	357	1081	1209	1307

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Days at room temp. in the dark	0	0	2	7	14	28	55	98
Extract volume for exposure ( $\mu\text{l}$ )	200	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>	100 <sup>a</sup>
<b>Dry feed</b> blank	tox	-76	-82	-70	-73	-79	-54	-74
E2 $\beta$ 5 ng g <sup>-1</sup>	tox	77	77	213	201	213	138	171
EE2 5 ng g <sup>-1</sup>	tox	51	80	193	198	232	141	173
DES 10 ng g <sup>-1</sup>	tox	79	81	148	165	213	109	111
zearalenone 1.25 $\mu\text{g g}^{-1}$	tox	46	58	221	229	276	93	411
equol 200 $\mu\text{g g}^{-1}$	tox	145	151	478	466	550	232	329
Days at room temp. in the dark	0	2	7	14	28	55	98	
Extract volume for exposure ( $\mu\text{l}$ )	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	
<b>Milkreplacer</b> blank		-16	5	-8	-14	-18	-15	-13
E2 $\beta$ 5 ng g <sup>-1</sup>		258	335	257	626	629	333	574
EE2 5 ng g <sup>-1</sup>		242	286	547	627	635	360	590
DES 10 ng g <sup>-1</sup>		246	360	232	576	594	356	544
zearalenone 1.25 $\mu\text{g g}^{-1}$		174	242	194	439	582	240	300
equol 200 $\mu\text{g g}^{-1}$		199	317	320	607	755	331	444
Days at -20 °C	0	2	7	14	28	55	98	
Extract volume for exposure ( $\mu\text{l}$ )	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	200 <sup>b</sup>	
<b>Wet Feed</b> blank		-1	-1	28	-1	-8	379	-16
E2 $\beta$ 5 ng g <sup>-1</sup>		313	487	363	682	719	403	588
EE2 5 ng g <sup>-1</sup>		309	377	417	679	772	477	546
DES 10 ng g <sup>-1</sup>		309	455	433	658	748	477	586
zearalenone 1.25 $\mu\text{g g}^{-1}$		261	304	329	528	744	434	381
equol 200 $\mu\text{g g}^{-1}$		264	342	285	638	812	-15	430



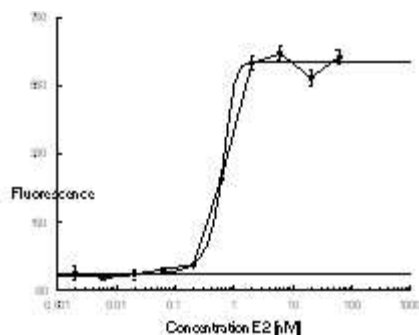
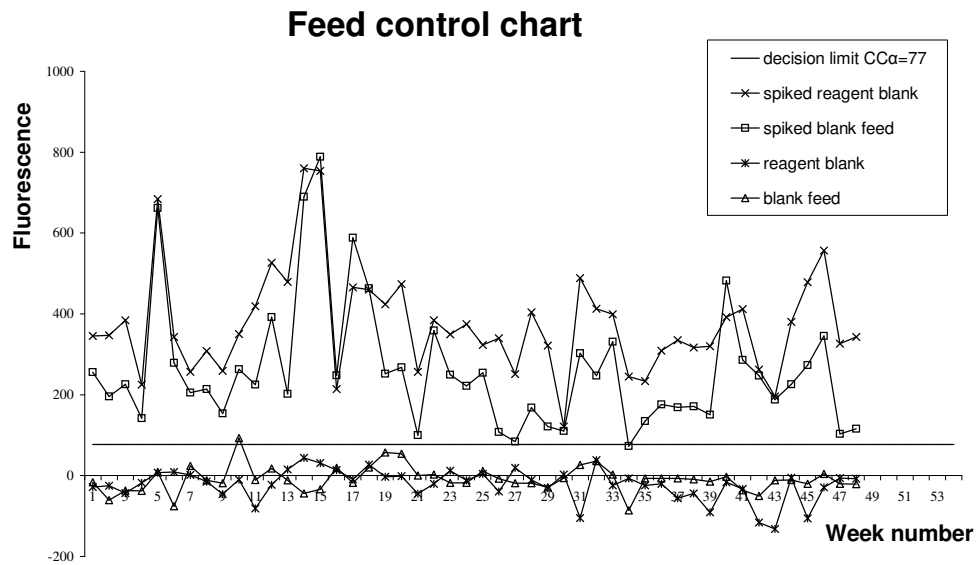


Figure 1. Response of the yeast estrogen cytosensor after a 24 h exposure to  $17\beta$ -estradiol. Exposure was started by adding 2  $\mu$ l of a  $17\beta$ -estradiol stock solution in DMSO to 200  $\mu$ l of a yeast culture. Fluorescence was determined after 0 and 24 hours as described in Experimental (see section 2.3 Yeast estrogen bioassay). Fluorescence signals are the mean of a triplicate with sd and are corrected for the signals obtained at 0 h and a reagent blank.

CONTROLEKAART RSV A1008 Veevoeder - Screening op estrogene activiteit - Gist bioassay met Fluorescentiemeting

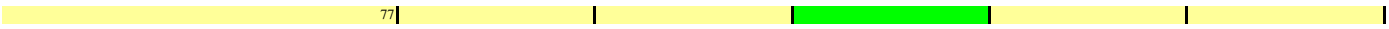


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Datum	afkeurgrens	Uitslag controlemonsters		t.o.v. blanco DMSO max ijklijn	t.o.v. blanco gist blanco chem	blanco voer	rest t.o.v. blanco chem opm.	
		blanco chem + 5 ng/g	blanco voer + 5 ng/g					
4/mei/04	77	345	256	653	-28	-16	10 ppb en 100ul van 2 ml	
13/jul/04	77	347	196	607	-25	-61		
13/jul/04	77	384	226	607	-43	-37		
21/sep/04	77	225	142	389	-18	-38		
18/okt/04	77	684	662	895	7	9		
25/okt/04	77	343	279	570	9	-76		5 ppb en 100 µl van 2 ml
11/nov/04	77	257	205	799	2	24		
11/nov/04	77	308	214	799	-15	-12	(10 ppb en 100ul van 2 ml)	
23/nov/04	77	259	154	807	-46	-19		
29/nov/04	77	350	263	683	-10	93		
6/dec/04	77	419	225	449	-81	-11		
13/dec/04	77	527	392	596	-23	18	5 ppb en 200 µl van 3 ml	
17/jan/05	77	479	202	485	15	-12		
31/jan/05	77	760	690	1039	44	-44		
31/jan/05	77	754	789	1039	31	-34		
7/feb/05	77	214	248	938	14	19		
22/feb/05	77	466	588	1026	-9	-18		
7/mrt/05	77	460	463	407	27	20		
21/mrt/05	77	424	252	765	-3	57		
21/mrt/05	77	474	268	765	-1	54		
4/apr/05	77	257	100	760	-45	0		
19/apr/05	77	384	359	1076	-20	3		
25/apr/05	77	349	250	504	12	-18		
25/apr/05	77	375	222	504	-12	-18		
2/mei/05	77	323	254	1022	4	11		
23/mei/05	77	340	108	748	-39	-8		
14/jun/05	77	251	84	898	19	-19		
4/jul/05	77	404	168	1112	-11	-19		
18/jul/05	77	322	121	762	-31	-29		
19/sep/05	77	121	110	1293	3	-6		wissel-ijklijn
22/sep/05	77	489	303	949	-105	26		wissel-ijklijn
22/sep/05	77	413	247	949	38	36		wissel-ijklijn
23/sep/05	77	399	331	484	-24	3		
26/sep/05	77	245	73	700	-7	-86		wissel-ijklijn,gekleurd extract, voer
18/okt/05	77	234	135	759	-24	-7		
18/okt/05	77	309	176	759	-20	-7		
18/okt/05	77	335	169	759	-56	-7		
19/okt/05	77	317	171	759	-44	-9		
19/okt/05	77	320	151	759	-91	-15		
19/okt/05	77	392	482	759	-16	-3		
27/okt/05	77	412	286	839	-33	-36		
27/okt/05	77	262	247	839	-116	-50		
27/okt/05	77	196	188	839	-132	-12		
27/okt/05	77	380	226	839	-5	-11		
27/okt/05	77	478	273	839	-106	-20		
27/okt/05	77	557	345	839	-29	4		
27/okt/05	77	326	103	839	-6	-20		
27/okt/05	77	343	116	839	-8	-21		
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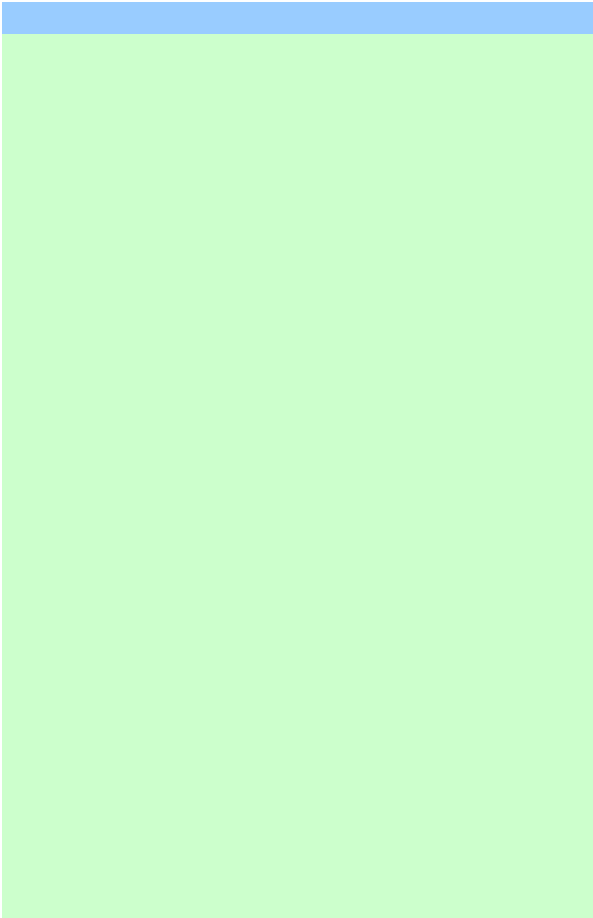
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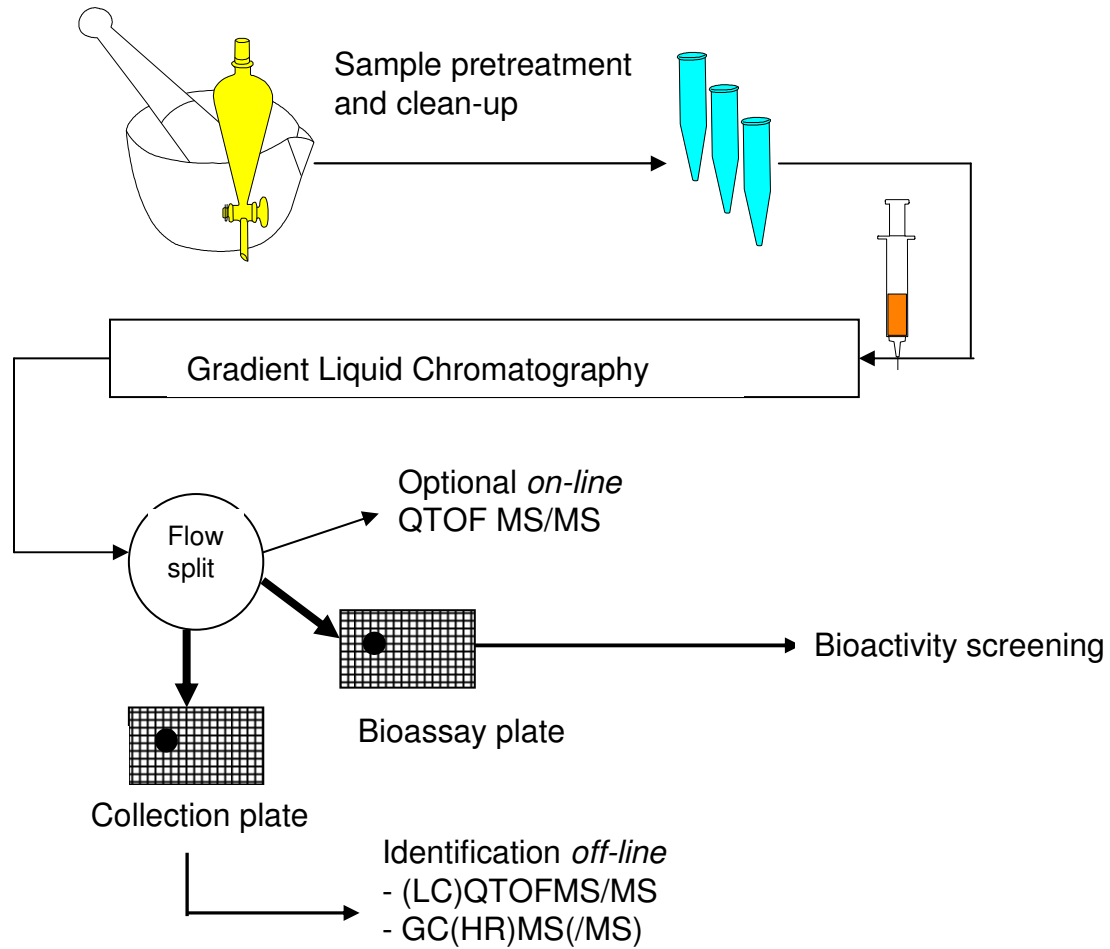
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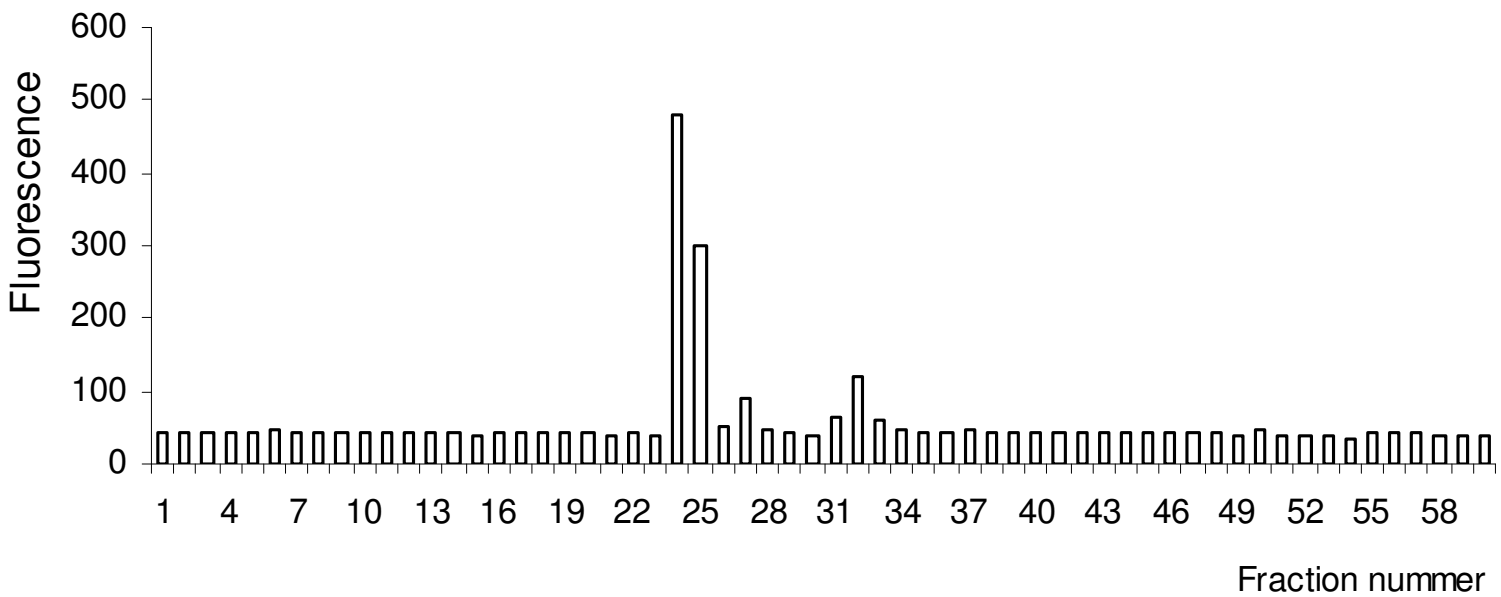




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WAARNEMINGENFORMULER GIST BIO-ASSAY ESTROGENEN	PAG. 1	DATUM	10/29/2004	LAB/JOURNAAL	XXXX1427	RUWE DATA FILE T0	rea4490
FRACTIEVERZAMELAAR	PLAATnr.	REA44-5	LOGBOEK			RUWE DATA FILE T24	rea44524
VERSIE: UITVANG HPLC-MS/MS SYSTEEM			RUWE DATA MAP	READ8		MS-FILE	

PPETTERSCHEMA MONSTER RIK131926

	1	2	3	4	5	6	7	8	9	10	11	12
1	FRACTIE 1	FRACTIE 16	FRACTIE 17	FRACTIE 32	FRACTIE 33	FRACTIE 48	FRACTIE 49	FRACTIE 64	FRACTIE 65	Blanco Gist	Blanco Gist	Blanco Gist
2	FRACTIE 2	FRACTIE 15	FRACTIE 18	FRACTIE 31	FRACTIE 34	FRACTIE 47	FRACTIE 50	FRACTIE 63	FRACTIE 66	Blanco Gist	600 µM E2B	Blanco Gist
3	FRACTIE 3	FRACTIE 14	FRACTIE 19	FRACTIE 30	FRACTIE 35	FRACTIE 46	FRACTIE 51	FRACTIE 62	FRACTIE 67	Blanco Gist	600 µM E2B	Blanco Gist
4	FRACTIE 4	FRACTIE 13	FRACTIE 20	FRACTIE 29	FRACTIE 36	FRACTIE 45	FRACTIE 52	FRACTIE 61	FRACTIE 68	Blanco Gist	600 µM E2B	Blanco Gist
5	FRACTIE 5	FRACTIE 12	FRACTIE 21	FRACTIE 28	FRACTIE 37	FRACTIE 44	FRACTIE 53	FRACTIE 60	FRACTIE 69	Blanco Gist	Blanco Gist	Blanco Gist
6	FRACTIE 6	FRACTIE 11	FRACTIE 22	FRACTIE 27	FRACTIE 38	FRACTIE 43	FRACTIE 54	FRACTIE 59	FRACTIE 70	Blanco Gist	Blanco Gist	Blanco Gist
7	FRACTIE 7	FRACTIE 10	FRACTIE 23	FRACTIE 26	FRACTIE 39	FRACTIE 42	FRACTIE 55	FRACTIE 58	Blanco Gist	Blanco Gist	Blanco Gist	Blanco Gist
8	FRACTIE 8	FRACTIE 9	FRACTIE 24	FRACTIE 25	FRACTIE 40	FRACTIE 41	FRACTIE 56	FRACTIE 57	Blanco Gist	Blanco Gist	Blanco Gist	Blanco Gist

RUWE DATA

T0	1	2	3	4	5	6	7	8	9	10	11	12
99	100	99	99	105	103	102	105	99	102	97	101	
99	107	99	99	104	101	100	101	99	97	98	100	
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96	98	98	98	103	101	101	101	99	97	99	98	
96	100	95	97	100	102	100	100	96	98	94	96	
97	100	100	96	99	100	99	97	95	96	94	97	
98	102	98	95	97	98	95	98	94	97	98	99	
120	122	96	95	98	100	101	98	99	105	99	102	

T24	1	2	3	4	5	6	7	8	9	10	11	12
395	387	383	374	396	383	377	383	370	373	367	372	
393	370	389	377	390	387	373	367	363	363	439	369	
399	356	394	378	383	389	364	364	358	364	427	369	
384	389	395	382	395	385	366	361	359	364	403	369	
384	389	391	380	370	374	367	365	362	359	356	363	
418	386	638	370	375	372	356	354	353	356	351	357	
408	396	626	373	380	373	358	359	346	357	364	361	
453	457	413	380	384	382	365	368	357	373	367	379	

BEREKENING T24 - T0 SIGNAAL

	1	2	3	4	5	6	7	8	9	10	11	12
296	287	284	275	291	280	275	278	271	271	270	271	
294	283	290	278	286	286	273	266	264	266	341	269	
302	259	294	274	281	289	259	262	261	263	332	269	
298	291	297	284	292	284	265	260	260	267	304	271	
298	289	296	283	270	272	267	265	266	261	262	267	
321	286	538	274	276	272	257	257	258	260	257	260	
310	294	528	278	283	275	263	261	252	260	268	262	
333	335	317	285	286	282	264	270	258	268	268	277	

CONTROLES

CONTROLE	GEMIDDELD	STDEV	BLANCO CORRECTIE
Blanco Gist	262	6	
600 µM E2	326	19	63

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	UITSLAG	CRITERIUM	VOLDOET	1e VALIDATIE	2e VALIDATIE

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WAARNEMINGENFORMULIER GIST BIO-ASSAY ESTROGENEN	PAG. 2	DATUM	10/29/2004	LAB/JOURNAAL	XXXX1427	RUWE DATA FILE T0	res04490
	PLAATnr.	REA44-5	LOGBOEK MS			0 RUWE DATA FILE T24	res044524
VERSIE: UITVANG HPLC-MS/MS SYSTEEM		RUWE DATA MAP	READS	MS-FILE			0

RESULTAAT

FRACTIE NR.	UITVANGVENSTER		RESULTAAT	CORRECTIE	UTSLAG
	VAN	TOT			
FRAC TIE 1	0.00	0.33	296	34	
FRAC TIE 2	0.33	0.66	294	32	
FRAC TIE 3	0.66	1.00	302	40	
FRAC TIE 4	1.00	1.33	298	36	
FRAC TIE 5	1.33	1.66	298	36	
FRAC TIE 6	1.66	2.00	321	59	RANDEFFECT??: HOGE OD
FRAC TIE 7	2.00	2.33	310	48	RANDEFFECT??: HOGE OD
FRAC TIE 8	2.33	2.66	333	71	
FRAC TIE 9	2.66	3.00	335	73	
FRAC TIE 10	3.00	3.33	294	32	
FRAC TIE 11	3.33	3.66	286	24	
FRAC TIE 12	3.66	4.00	289	27	
FRAC TIE 13	4.00	4.33	291	29	
FRAC TIE 14	4.33	4.66	259	-3	
FRAC TIE 15	4.66	5.00	263	1	
FRAC TIE 16	5.00	5.33	287	25	
FRAC TIE 17	5.33	5.66	284	22	
FRAC TIE 18	5.66	6.00	290	28	
FRAC TIE 19	6.00	6.33	294	32	
FRAC TIE 20	6.33	6.66	297	35	
FRAC TIE 21	6.66	7.00	296	34	
FRAC TIE 22	7.00	7.33	538	276	VERDACHT , BEVAT MOGELIJK 17-B ESTRADIOL
FRAC TIE 23	7.33	7.66	528	266	VERDACHT , BEVAT MOGELIJK 17-B ESTRADIOL
FRAC TIE 24	7.66	8.00	317	55	
FRAC TIE 25	8.00	8.33	285	23	
FRAC TIE 26	8.33	8.66	278	16	
FRAC TIE 27	8.66	9.00	274	12	
FRAC TIE 28	9.00	9.33	283	21	
FRAC TIE 29	9.33	9.66	284	22	
FRAC TIE 30	9.66	10.00	274	12	
FRAC TIE 31	10.00	10.33	278	16	
FRAC TIE 32	10.33	10.66	275	13	
FRAC TIE 33	10.66	11.00	291	29	
FRAC TIE 34	11.00	11.33	286	24	
FRAC TIE 35	11.33	11.66	281	19	
FRAC TIE 36	11.66	12.00	292	30	
FRAC TIE 37	12.00	12.33	270	8	
FRAC TIE 38	12.33	12.66	276	14	
FRAC TIE 39	12.66	13.00	283	21	
FRAC TIE 40	13.00	13.33	286	24	
FRAC TIE 41	13.33	13.66	282	20	
FRAC TIE 42	13.66	14.00	275	13	
FRAC TIE 43	14.00	14.33	272	10	
FRAC TIE 44	14.33	14.66	272	10	
FRAC TIE 45	14.66	15.00	284	22	
FRAC TIE 46	15.00	15.33	289	27	
FRAC TIE 47	15.33	15.66	286	24	
FRAC TIE 48	15.66	16.00	280	18	
FRAC TIE 49	16.00	16.33	275	13	
FRAC TIE 50	16.33	16.66	273	11	
FRAC TIE 51	16.66	17.00	259	-3	
FRAC TIE 52	17.00	17.33	265	3	
FRAC TIE 53	17.33	17.66	267	5	
FRAC TIE 54	17.66	18.00	257	-5	
FRAC TIE 55	18.00	18.33	263	1	
FRAC TIE 56	18.33	18.66	264	2	
FRAC TIE 57	18.66	19.00	270	8	
FRAC TIE 58	19.00	19.33	261	-1	
FRAC TIE 59	19.33	19.66	257	-5	
FRAC TIE 60	19.66	20.00	265	3	
FRAC TIE 61	20.00	20.33	260	-2	
FRAC TIE 62	20.33	20.66	262	0	
FRAC TIE 63	20.66	21.00	266	4	
FRAC TIE 64	21.00	21.33	278	16	
FRAC TIE 65	21.33	21.66	271	9	
FRAC TIE 66	21.66	22.00	264	2	
FRAC TIE 67	22.00	22.33	261	-1	
FRAC TIE 68	22.33	22.66	260	-2	
FRAC TIE 69	22.66	23.00	266	4	
FRAC TIE 70	23.00	23.43	258	-4	

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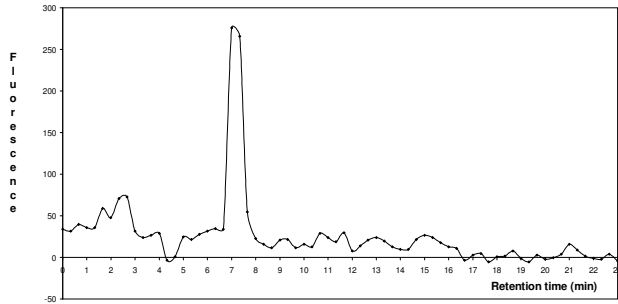
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WAARNEMINGENFORMULIER GIST BIO-ASSAY ESTROGENEN	PAG. 3	DATUM	10/29/2004	LAB/JOURNAAL	XXXX1427	RUWE DATA FILE T0	rea4490
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