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A RIKILT yeast Estrogen bioAssay (REA) for estrogen residue detection in urine of calves experimentally treated with 17beta-estradiol.

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3 1 **A RIKILT yeast Estrogen bioAssay (REA) for estrogen residue detection in**
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6 2 **urine of calves experimentally treated with 17 β -estradiol.**
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3 20 **Abstract**
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5 21 17 β -estradiol is one of the most powerful sex steroids illegally used in bovine production. The
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8 22 objective of this study was to evaluate the application and the specificity of the RIKILT yeast
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10 23 Estrogen bioAssay (REA) for the detection of molecules with estrogenic activities in the urine of
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13 24 calves experimentally treated with anabolics. Four groups of six calves each received an injection of
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15 25 17 β -estradiol i.m. (group B), androsterone and gliburide (group A), and testosterone (group C)
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18 26 molecules at different dosage for 40 days. Group D was the control. The ability of the REA test to
19
20 27 detect estrogenic activity in urine samples from all animals was assessed. All estrogen-treated
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22 28 animals (group B) showed as being positive up to seven days after administration of the highest
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24
25 29 dosage of 17 β -estradiol, while the other three groups showed as being negative. The identity of
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27 30 estrogenic molecules in the urine of group B (17 β -estradiol, 17 α -estradiol, and estrone) was
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30 31 confirmed by GS/MS. This is the first time the REA test has been applied to detect 17 β -estradiol in
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32 32 the urine of calves treated with the hormone *in vivo*. The technique may offer an advantageous
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35 33 laboratory method for the veterinary surveillance of illegal steroid use.
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35 Introduction

36 European Union (EU) regulations ban the use of growth promoters in meat-producing animals
37 (European Commission 1996a), however their illegal use raises cause for concern. By acting on
38 lipid and protein metabolism, anabolic steroids increase growth rate, stimulate muscle growth, and
39 reduce fat tissue in calves (Groot *et al* 1998). One of the most efficient of these anabolics is
40 17β -estradiol. Histologically, it induces hyperplasia and metaplasia of prostate epithelial cells and
41 urethral epithelium (Ruitenber *et al.*, 1970; Kroes *et al.*, 1971; Groot *et al.*, 1998). Squamous
42 metaplasia is a specific response to a variety of estrogenic substances such as estradiol,
43 diethylstilbestrol (DES) and zeranol, and it is considered as a reliable histological marker to assess
44 their estrogenic action in cattle.

46 But for surveillance and monitoring of the ban to be effective, validated analytic methods that can
47 screen for and confirm hormone residues in urine and other sample matrices are needed (European
48 Commission 1996b, European Commission 2002), as is knowledge of the absorption,
49 biotransformation, and excretion kinetics of illegally administered hormones and the levels of
50 endogenous hormones in these matrices. As current official techniques seem to be unsatisfactory
51 (Groot *et al* 2007), new simple methods need to be developed and implemented alongside
52 conventional veterinary controls.

54 Recent scientific data have implicated estrogen in tumor progression of human breast and uterine
55 cancer, where it acts as a hormone stimulating cell proliferation, and as a procarcinogen agent that
56 induces genetic damage (Liehr, 2000; Subramanian A. *et al.*, 2008; Foster P.A., 2008). Methods
57 that can monitor estrogen residues in animal foods and derivatives and reduce the risk of
58 contamination are therefore urgently required.

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3 60 Various techniques that can detect estrogenic substances include gas chromatography-mass
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5 61 spectrometry (GC-MS) (Daeseleire *et al.*, 1992; Fotsis and Adlercreutz, 1987), high-performance
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7 62 liquid chromatography-mass spectrometry (HPLC-MS) (Sorensen and Elbaek, 2005) and
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10 63 immunoassay (Dixon and Russell, 1983; Jansen *et al.*, 1986; Chen *et al.*, 1996).
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12 64
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15 65 Despite the limited number of positives found in controls, findings and analysis of illegal
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17 66 preparations show that steroids, natural hormones and β agonists are still being used (Courtheyn *et*
18
19 67 *al.*, 2002; Nielen *et al.*, 2003). The analytical methods for measuring residues of steroids and β -
20
21 68 agonists as used in control programs are unable to detect very new or outdated compounds and
22
23 69 might be one of the possible explanations for the limited positives found so far. Screening and
24
25 70 confirmatory GC-MS, and even the multi-residue liquid chromatography tandem-mass spectrometry
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27 71 (LC/MS/MS) methods, are limited to a short list of a priori known hormone residues (Cai *et al.*,
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29 72 1997; Hewitt *et al.*, 2002; Van Poucke and Van Peteghem, 2002). The multi-analyte screening
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31 73 ability of radio- and enzyme immunoassays depends on the limited degree of cross-reactivity of the
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33 74 antibody used (Vanoosthuyze *et al.*, 1997; Haasnoot *et al.*, 2002). Alternatively, receptor-based
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35 75 assays can be used to detect compounds having affinity for a given receptor (Mueller, 2002);
36
37 76 specifically, yeast-based assays feature several advantages, including robustness, low cost, lack of
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39 77 known endogenous receptors, and the use of media devoid of steroids (Breithofer *et al.*, 1998;
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41 78 Graumann *et al.*, 1999; Witters *et al.*, 2001).
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51 80 Recently, Bovee *et al.* (2004a, 2004b) developed a bioassay called RIKILT yeast Estrogen
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53 81 bioAssay (REA), which is fully validated according to the guidelines as prescribed in EC Decision
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55 82 2002/657 and accredited ISO 17025. The assay consists of a recombinant yeast cell that stably
56
57 83 expresses the human estrogen receptor α (ER α) and a yeast-enhanced green fluorescent protein
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59 84 (yEGFP) as a reporter protein in response to estrogens. The GFP is a protein that exhibits green
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85 fluorescence that can be measured directly (Cormack *et al.*, 1997). The yEGFP can be measured

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3 86 much more easily than an extra-chromosomal reporter construct with β galactosidase as a substrate-
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6 87 based reporter protein, a system frequently used by older yeast-based assays (Routledge and
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8 88 Sumpter, 1997; Gaido *et al.*, 1997; Rehmann *et al.*, 1999; Morito *et al.*, 2001; Le Guevel and
9
10 89 Pakdel, 2001).

11
12
13 90
14
15 91 The EC50 revealed by the REA test was 0.5 nM for 17 β -estradiol and was comparable with
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17
18 92 reporter EC50 values for yeast estrogen bioassays that contain β galactosidase as a reporter (Bovee
19
20 93 *et al.*, 2004c). The yEGFP assay can be performed completely in 96-well plates within 24 h and
21
22
23 94 does not need cell wall disruption nor the addition of a substrate. Finally, the REA test is extremely
24
25 95 robust and survives extracts from dirty sample matrices such as urine and feed and it is able to
26
27 96 recognize estrogen molecules (natural and artificial hormones, aflatoxin and phytoestrogens) (Bovee
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30 97 *et al.*, 2004b)

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34 99 This bioassay is routinely applied as a qualitative screening method in both calf urine and feed
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37 100 sample matrices in Dutch laboratories: only sample extracts showing a fluorescence response
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39 101 beyond the decision limit $CC\alpha$ will be declared suspect for estrogen activity. Subsequently, the
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42 102 identity of substances causing estrogenicity must be determined by conventional residue analytical
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44 103 methods such as GC-MS or by using bioassay-directed quadrupole time-of-flight MS (QTOFMS)
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46 104 identification approaches (Nielen *et al.*, 2004, 2006). Moreover, the bioassay could be a good tool
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49 105 alongside instrumental analysis for determining estradiol residues in bovine urine.

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51 106
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53 107 The aim of this study was to evaluate the ability of the REA to detect estradiol residues in the urine
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56 108 of experimentally treated veal calves, and to compare the results with those from official analytical
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58 109 methods (GC/MS).

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110 **Material and methods**

111 Animals and experimental design

112 Twenty-four cross-bred Friesian male calves six months old were randomly assigned to four
113 experimental groups: group A (n=6) treated daily with 1ml/animal of gliburide (0.2 mg/L) and
114 androsterone (8 mg/L) cocktail i.m. (Krentz and Bailey, 2005); group B (n=6) treated weekly with 17 β -
115 estradiol (diluted in 10 ml of benzylic alcohol and 1 ml of ethyl oleate) i.m. six times until one
116 week before slaughter, for a total of 190 mg/animal (Table 1); group C (n=6) treated weekly with
117 testosterone propionate (diluted in sesame oil) i.m. , for a total of 1.050 g/animal (Table 1). Group
118 D (n=6) was the control.

119 The entire treatment phase lasted for a total of 44 days.

120
121 The average body weight of the animals at the start of the animal housed was approximately 140
122 kg; the calves were tethered and fed with liquid milk replacer twice a day (providing per kg: dry
123 matter (DM) 950 g, crude protein (CP) 230 g, ether extract (EE) 210 g, ash 60 g, cellulose 1 g,
124 retinol 75 mg, ascorbic acid 50 mg, Cu 5 mg, cholecalciferol 0.125 mg, alphotocopherol 80 mg).

125 The amount of the fodder was increased gradually up to 8 l/calf/day, then increased gradually up to
126 16 l/calf/day; after 1 month, a quantity of 0.5 kg of barley straw (per kg: DM 900 g, CP 20 g, EE 10
127 g, ash 60 g, crude fiber 410 g) was added to the diet according to the indications suggested by the
128 European Commission (97/182/EC). The experiment was authorized by the Italian Ministry of
129 Health and the Ethical Committee of the University of Turin. The carcasses of the treated animals
130 were destroyed (2003/74/CE - DL 16 March 2006, No. 158).

131
132 Urine samples of experimental group B were collected at 5, 24, 48 and 168 h after the injection of
133 estradiol (I, III, and VI doses), analyzed by REA and then confirmed by GC-MS. Urine samples
134 from the experimental groups (A, C) were analyzed with the REA test at 15 and 44 days into the

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3 135 study. All urine samples from each experimental group were compared with the fluorescence data
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6 136 obtained from the control animals.

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10 138 Steroid extraction of calf urine samples

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13 139 Aliquots of 2 ml of calf urine of each experimental group were adjusted to pH 4.8 and 6 μl β -
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15 140 glucuronidase/arylsulfatase (300 and 22.5 Uml^{-1} , respectively) were added. Enzymatic
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18 141 deconjugation was carried out overnight in a water bath at 37°C. Next, 2 ml of 0.25 M sodium
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20 142 acetate buffer, pH 4.8, was added and the hydrolysed sample was subjected to solid-phase
21
22 143 extraction (SPE) on a C18 column fitted on a vacuum manifold (Supelco). The column was
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24
25 144 previously conditioned with 2.5 ml methanol and 2.5 ml sodium acetate buffer. The column was
26
27 145 then washed with 1.5 ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5 ml sodium acetate
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30 146 buffer, pH 4.8, 3.0 ml water, and finally with 2 ml methanol/water (50:50, v/v). The column was
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32 147 air-dried and eluted with 4 ml acetonitrile, which was applied to an NH₂-column (Supelco)
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34 148 previously conditioned with 3.0 ml acetonitrile. The acetonitrile eluate was evaporated to 2 ml
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36
37 149 under a stream of nitrogen gas. A 100- μl aliquot of this extract (equivalent to 100 μl urine) was
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39 150 transferred to a 96-well plate in triplicate and 50 μl water and 2 μl DMSO were added to each well.
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42 151 To remove the acetonitrile from this mixture, the plate was dried overnight in a fume cupboard and
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44 152 was then ready to be screened for estrogenic activities with the yeast estrogen bioassay.

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49 154 REA

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51 155 The yeast cytosensor expressing the human estrogen receptor α (hER α) and yeast-enhanced green
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54 156 fluorescent protein (yEGFP) in response to estrogens has been described previously (Bovee *et al.*,
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56 157 2004a, b). The yeast growth was performed according to published data (Bovee *et al.* 2004a). For
57
58 158 exposure in 96-well plates, 200- μl aliquots of this yeast culture were pipetted into each well
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159 already containing the extracts of the urine samples. A 17 β -estradiol dose-response curve in

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3 160 DMSO was included in each exposure experiment (Fig. 1). Each urine sample extract and each
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6 161 17 β -estradiol standard was assayed in triplicate. Exposure of the yeast to the sample was
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8 162 performed 24 h and the yeast fluorescence at 0 h and 24 h was measured directly in a Victor
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11 163 31420 Multilabel Counter (PerkinElmer) using excitation at 485 nm and emission measurement at
12
13 164 530 nm. Differences of the fluorescence measurement at 24 h and 0 h of yeast exposed to samples
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15 165 (t_{24-t_0}) were calculated and corrected for the reagent blank data, thus providing the final estrogenic
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18 166 activity data for each sample. The samples are reported 'suspect' when the t_{24-t_0} fluorescence
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20 167 measurement exceeds the CC α (fluorescence of 4409) obtained in the ring test in our laboratory at
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23 168 the University of Turin, Italy (Bovee *et al.*, 2008); otherwise, the samples are reported 'compliant'.
24
25 169 The bioassay is a qualitative screening method, i.e. no concentrations are reported. We have
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27 170 analyzed a total of 114 samples.
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30 171 31 32 172 GC/MS analysis

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34 173 The deconjugated estrogens were extracted from 2 mL of aqueous samples, derivatized and
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37 174 analyzed with an Agilent Technologies 6890 N GC connected to an Agilent 5975 MSD (Akre *et*
38
39 175 *al.*, 2004; Mouatassim-Souali *et al.*, 2003; Daeseleire *et al.*, 1998). The internal standard was
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41 176 methyl testosterone at a final concentration of 75 ng/mL. In order to hydrolyze the glucurono-
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43
44 177 conjugate of 17 α - and 17 β -estradiol, the pH was adjusted between 6.5 to 7 with HCl 1 M; 50 μ L
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46 178 of enzyme β -glucuronidase (441 I.U.) were added and hydrolyzed for 1 h at 42°C. We extracted
47
48
49 179 17 α - and 17 β -estradiol at pH 9 for NaOH 1M by liquid/liquid extraction with 10 mL of tert-butyl-
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51 180 methyl-ether. The organic phase was dried under nitrogen stream at 50°C. The analytes were
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53
54 181 derivatized with 50 μ L of tetramethylsilane and heated to 70°C for 30 min in thermoblock.
55
56 182 Compounds of interest were separated on a HP-5 column (17 m x 0.20 mm x 0.33 μ m film
57
58
59 183 thickness). The temperature program was 90°C for 1.50 min, ramped at 25°C/min to 260°C and
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184 held for 3 min, ramped at 25°C/min to 320 °C and held for 1.30 min. A 2- μ l aliquot was injected

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3 185 into the GC–MS system in splitless mode. The injector port temperature was 280°C, the transfer
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6 186 line from the GC system to the mass-selective detector was held at 280°C. Helium was the carrier
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8 187 gas at 2.1 mL/min initial flow. The pressure was constant. The mass-selective detector was operated
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10 188 in electronic impact mode using selected ion monitoring (SIM).

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15 190 Selection of diagnostic ions was carried out after acquisition of complete mass spectra for analyte,
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17 191 both alone and in the working standard, under the same chromatographic conditions as for the
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20 192 samples. The masses for 17 α - and 17 β -estradiol were $m/z = 416$, $m/z = 401$, $m/z = 326$, $m/z = 285$,
21
22 193 $m/z = 232$, and $m/z = 446$ and $m/z = 301$ for methyl testosterone. The dwell time was 80 ms. The
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24
25 194 retention times were 11.80 min for 17 α -estradiol, 12.20 min for 17 β -estradiol, and 12.80 min for
26
27 195 internal standard. Calibration curves were constructed by extracting urine spiked with 17 α - and
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29
30 196 17 β -estradiol at a final concentration of 5, 10, 30, 50 and 100 ng/mL. The calibration graph for each
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32 197 analyte is described by the equation $Y = mX + b$, whereby Y represents the ratio between the
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34
35 198 response value of the analyte (peak area) and the response value of the internal standard (peak area),
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37 199 and X is the concentration in ng/mL of analyte in the sample. We have analyzed a total of 104
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39 200 samples.

40 41 42 201 43 44 202 Statistical analysis

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46 203 The validity of the REA test was evaluated by comparing the test results with those of the GC-MS
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49 204 method which represents the “golden test” or “gold standard” (Petrie and Watson, 2006). Validity
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51 205 criteria were sensitivity, specificity and concordance index or efficiency, which is the concordance
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53 206 degree of the test. Evaluation was easily done using a 2x2 contingency table and the sensitivity (%)
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56 207 was calculated as $100 \times \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false}}$
57
58 208 negatives), the specificity (%) as $100 \times \frac{\text{number of true negatives}}{\text{number of false positives} +}$
59
60 209 $\text{number of true negatives}$), and the concordance index (%) as $100 \times \frac{\text{number of true positives} +}$
210 $\text{number of true negatives}}{\text{total number of samples}}$.

211 Results

212 Figure 1 shows the dose-response-curve obtained with the REA test after 24-h exposure to
213 17 β -estradiol. Exposure was started by adding 2 μ l of a 17 β -estradiol stock solution in DMSO to
214 200 μ l of a yeast culture. The signals are the responses obtained after 24 h of exposure and are
215 corrected with the responses obtained at 0 h ($t_{24}-t_0$) and with the response ($t_{24}-t_0$) obtained with a
216 reagent blank (DMSO). All the responses are reported as the means of a triplicate.

217 After 24 h of yeast exposure with the different 17 β -estradiol concentration (standards), we didn't
218 found any differences in the yeast growth, meaning that no toxic effects on the yeast could be
219 observed (data not shown).

220
221 Figure 2 illustrates the trends of REA response in calf urine samples collected at different times and
222 after administration of different doses. As shown in Figures 2a and 2b, all urine samples collected at
223 5 and 24 h after the first and third dose of 17 β -estradiol showed a higher fluorescence than the
224 decision limit $CC\alpha$ (fluorescence 4409) (Bovee *et al.*, 2008). Five out of six urine samples collected
225 after the first dose and four out of six samples collected 48 h after the third dose showed
226 fluorescence over 4409 (Fig. 2a and 2b). After one week, all urine samples from animals that had
227 received the first and third dose showed a fluorescence lower than the $CC\alpha$. Instead, at the sixth
228 dose all the 17 β -estradiol-treated animals (Fig. 2c) had fluorescence data higher than 4409 also
229 after 168 h.

230
231 Table 2 compares the data from the REA test and the GC-MS analysis. The GC-MS results of all
232 urine samples after administration of dose I at T0 demonstrate that endogen 17 α -estradiol and
233 17 β -estradiol were absent (sensitivity of method was 1 ppb). At 5 h after the first dose a small
234 quantity of 17 β -estradiol and a significant increase in its metabolite (17 α -estradiol) was detectable
235 in the animals. After 24 h, the 17 β -estradiol level began to decrease, whereas the 17 α -estradiol

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3 236 level continued to rise. At 48 h after injection, 17 β -estradiol had been completely metabolized and
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6 237 converted into 17 α -estradiol.
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11 239 At one week (168 h) after the administration of the first dose, 17 β -estradiol was absent, except in
12
13 240 animal 301095 where traces of 17 α -estradiol were found. At T0 of the third dose no analytes were
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16 241 found, but an increase in both 17 α -estradiol and 17 β -estradiol was detectable after treatment.
17
18 242 REA test specificity was evaluated by comparing the urine samples from calves in groups A
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21 243 (cocktail treatment), C (testosterone treatment) and D (control animal); no animals were found
22
23 244 positive to estrogens (Table 3).
24
25 245
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27
28 246 Table 4 illustrates the 2x2 contingency table for evaluating the validity of the REA method as a
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30 247 qualitative screening test versus the results obtained with GC/MS. The REA test was found to have:
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32 248 $100 \times (83/(83+5))=94.3\%$ sensitivity, $100 \times (16/(0+16))=100\%$ specificity, and a concordance index
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35 249 of $100 \times (83+16)/104=95.2\%$
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37 250
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39 251 **Discussion**

41
42 252 Estrogens are among the most important growth promoters used illegally in animal production and
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44 253 their use is prohibited in the European Union (Council Directive 1996). An alternative approach to
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46 254 chemical analysis (Courtheyn *et al.*, 2002) for the identification of treated animals might be the use
47
48
49 255 of a bioassay. Bovee *et al.* (2004a, 2004b) developed a bioassay, (REA), fully validated according
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51 256 to the guidelines as prescribed in EC Decision 2002/657 and accredited ISO 17025. In The
52
53
54 257 Netherlands, the test is routinely applied as a screening test for the detection of illegal estrogen
55
56 258 treatment in calves.
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260 In 2006, Nielen *et al.* analyzed more than 120 calf urine samples randomly collected from different
261 Dutch calves, and compared the urine data obtained with the REA test with data derived from

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262 GC/MS/MS. The data for REA validation were obtained from aliquots of blank calf urine and calf
263 urine samples spiked with 17β -estradiol and other estrogen molecules. Currently, there are no data
264 on REA application in animals experimentally treated with estrogenic hormones.

265
266 In this study we evaluated the ability of REA to detect estradiol residues in the urine of
267 experimentally treated veal calves, and compared the results with an official analytical method
268 (GC/MS).

269
270 In hormone treatment of cattle, the urinary profile usually contains a mixture of metabolites of the
271 anabolic drug that was used. Since the bioassay provides an additive measurement of all estrogenic
272 residues and metabolites (having different relative estrogenic potencies), the chance of overlooking
273 illegal treatment is relatively low, as compared with other screening assays such as highly specific
274 immunoassays. In a previous study (Bovee *et al.*, 2004b), it was shown that 17β -estradiol
275 metabolites (17α -estradiol, estrone, estriol and hydroxylated estrogens) give a response in the
276 bioassay.

277
278 We demonstrated that GC/MS analysis of urine samples confirmed the results obtained with the
279 REA method, except for five samples of group B. Three real false negatives were: the sample
280 taken at 48 h after injection III in animal 290403, the sample taken at 72 h after injection VI in
281 animal 252732 and the sample taken at 0 h after injection III in animal 300408 resulting in a
282 percentage of false negatives that is below the accepted level of 5%.

283
284 The other two samples corresponded to animal 301095 collected at 48 and 168 h after the first
285 treatment.; they did not contain 17β -estradiol and contain small quantities of 17α -estradiol (6.29
286 and 2.52 ng/ml respectively) and resulted negative at REA. However, the 17α -estradiol metabolite
287 is about ten times less potent than 17β estradiol, the relative estrogenic potency (REP) of 17α -

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3 288 estradiol is 0.093 compared to 17 β -estradiol (Bovee *et al.*, 2004b). Moreover, the assay was
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6 289 validated on the 1 ppb 17 β -estradiol level, which is generally accepted as the limit in urine, and the
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8
9 290 assay was then shown to produce less than 5% of false negatives. This 1 ng 17 β -estradiol per ml
10
11 291 urine level corresponds to the activity of 10 ng 17 α -estradiol per ml. Thus it can be discussed
12
13 292 whether these screening results are real false negatives or not. In addition, Bovee *et al.* (2006)
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16 293 calculated that the limit for an increase in fluorescence of the REA test was about 5.5 ng of
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18 294 17 α -estradiol per ml urine, demonstrating that the negative screening outcome of the sample
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21 295 containing 2.52 ng 17 α -estradiol per ml was as expected, while the 6.29 ng 17 α -estradiol per ml
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23 296 sample is just above this limit and was screened as negative as well. However, our results (fig. 2a)
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25
26 297 showed an increased fluorescence response of the latter sample, but it was below the determined
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28 298 CC α value of 4409 (Bovee *et al.*, 2008).
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32
33 300 Finally, the outcome of the urine sample of animal 252732 at 48 h after the first dose of
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35 301 17 β -estradiol contained no 17 β -estradiol and 4.47 ng 17 α -estradiol per ml and was screened as
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38 302 suspect in estrogen bioassay. Once more demonstrating that the limit of detection is about 5.5 ng
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40 303 17 α -estradiol per ml, but this study also shows that all samples containing 1 ng 17 β -estradiol or
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43 304 more (equal to 10 ng 17 α -estradiol per ml or more) are screened as suspect. On the basis of these
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45 305 considerations, the last two samples couldn't be considered the real false negative. Unlike the REA
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47 306 test, which is a qualitative method, GC-MS showed fluctuations in estrogen levels of urine samples
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50 307 from different calves under the same experimental conditions, same time and dose; this fluctuation
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52 308 is likely due to variability in an animal's metabolism. Animal 301095 was noted to maintain a small
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54
55 309 quantity of 17 α -estradiol also at one week after the first dose probably because of greater difficulty
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57 310 in eliminating the metabolite. In calf 252732, on the other hand, 17 α -estradiol concentration
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60 311 rapidly peaked and was then quickly eliminated (Table 2).

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3 312 In conclusion, the animals can metabolize the first two treatments in a week, while subsequent
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6 313 doses, stronger, leaving significant residues of 17α -estradiol and less 17β -estradiol after one week
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8 314 to treatment. The 17α -estradiol is systematically present in concentration 10-50 times higher than
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11 315 the 17β -estradiol, index of quick conversion of one form in the other.
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15 317 Statistical analysis confirmed that the bioassay is a valid method that can discriminate between
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18 318 estrogen-treated and non-estrogen-treated calves when compared against the results of an official
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20 319 chemical test like the GC-MS. However, screening with this bioassay is limited to calf urine
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23 320 because older bovines produce large amounts of endogenous 17α -estradiol and estrone, which
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25 321 would cause too many false-suspect urine values.
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30 323 Sample preparation is quite similar in both methods, but the cost of this test and the easier
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32 324 interpretation of the REA results procedure make it a more practical method for large scale
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35 325 screening to support the chromatography method. Currently, it is not possible to discriminate
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37 326 natural estrogen molecules from synthetic estrogenic hormones illegally injected into animals.
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39 327 Furthermore, the REA method can be applied only to samples from prepuberal veal calves fed
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42 328 regular milk replacer. Nonetheless, the REA method is a fast and simple assay for routine
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44 329 surveillance and monitoring and it can significantly reduce the GC-MS sample workload.
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46 330 47 48 49 331 **ACKNOWLEDGMENTS.**

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60**Legends**

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467 Figure. 1. Response of REA after 24-h exposure to 17 β -estradiol. Exposure was started by adding 2
468 μ l of a 17 β -estradiol stock solution in DMSO to 200 μ l of a yeast culture. Fluorescence was
469 determined after 0 and 24 h as described in Experimental (see section on Yeast estrogen bioassay).
470 Fluorescence signals are the means \pm SD of a triplicate and are corrected for the signals obtained at
471 0 h and a reagent blank.

472
473 Figure 2. Results obtained by REA to 17 β -estradiol and metabolites from extract of urine samples
474 from each animal of group B treated with: a) 20mg/animal of 17 β -estradiol (I dose), b)
475 30mg/animal of 17 β -estradiol (III dose), c) 40mg/animal of 17 β -estradiol (VI dose). (◆) calf no.
476 297627, (■) calf no. 290403, (▲) calf no. 301095, (×) calf no. 301096, (△) calf no. 252732, (○)
477 calf no. 300408, (—) decision limit CC α (4409).

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2 1 **Tables**3
4 2 Table. 1. Treatment of group B and group C calves.
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Dose	Group B 17β-estradiol (mg/animal)	Group C Testosterone (mg/animal)
I	20	150
II	20	150
III	30	150
IV	40	200
V	40	200
VI	40	200

4 Table 2. 17 α - and 17 β -estradiol screening of urine samples by REA and GC-MS methods.

ID animal	Dose	Time from injection (h)	17 α -estradiol (ng/ml)	17 β -estradiol (ng/ml)	REA response	
301096	I	0	<1	<1	-	
		5	247.2	30.53	+	
		24	464.74	<1	+	
		48	98.68	<1	+	
		168	<1	<1	-	
	III	0	<1	<1	-	
		5	568	76	+	
		24	609	13	+	
		48	248	4.5	+	
		168	n.t.*	n.t.	-	
	VI	0	117	3.1	+	
		5	418	96	+	
		24	1356	80	+	
		48	582	23	+	
		72	239	8.7	+	
		96	23	<1	+	
		120	187.59	9.26	+	
		144	82.97	4.72	+	
		168	216.14	10.37	+	
		300408	I	0	<1	<1
	5			255.57	5.92	+
	24			294.91	22.02	+
	48			20.63	<1	+
	168			<1	<1	-
III	0		233.67	28.83	-	
	5		411.	34	+	
	24		399.65	20.84	+	
	48		155.85	7.36	+	
	168		n.t.	n.t.	-	
VI	0		20	<1	+	
	5		2202	273	+	
	24		525	19	+	
	48		111	4.8	+	
	72		129	3.6	+	
	96		183	8.7	+	
	120		96.03	4.29	+	
	144		26.54	<1	+	
	168		n.t.	n.t.	+	
	252732		I	0	<1	<1
5				156.04	1.45	+
24				238.25	<1	+
48				4.57	<1	+
168				<1	<1	-
III		0	<1	<1	-	
		5	401.35	62.5	+	
		24	704.99	23.28	+	
		48	180.67	5.09	+	

1					
2		168	n.t.	n.t.	-
3	VI	0	148	5.8	+
4		5	820	183	+
5		24	2022	112	+
6		48	420	20	+
7		72	72	1.8	-
8		96	100	4.6	+
9		120	n.t.	n.t.	+
10		144	100	2.3	+
11		168	67	3.8	+
12	301095	I	0	<1	-
13		5	341.62	67.74	+
14		24	865.94	29.63	+
15		48	6.29	<1	-
16		168	2.52	<1	-
17		III	0	<1	-
18		5	606.53	126.04	+
19		24	297.43	8.16	+
20		48	104.19	<1	+
21		168	n.t.	n.t.	-
22		VI	0	45.3	+
23		5	1142.03	369.12	+
24		24	765.91	59.08	+
25		48	141.34	<1	+
26		72	58	2	+
27		96	36	1.1	+
28		120	70.46	<1	+
29		144	16.11	<1	+
30		168	64	2.8	+
31	290403	I	0	<1	-
32		5	353	13	+
33		24	1601	41	+
34		48	74	<1	+
35		168	<1	<1	-
36		III	0	<1	-
37		5	637	137	+
38		24	684	18	+
39		48	176	6.0	-
40		168	n.t.	n.t.	-
41		VI	0	38.46	+
42		5	296.93	74.77	+
43		24	664.62	57.28	+
44		48	365.52	20.72	+
45		72	137	5.4	+
46		96	100	4.7	+
47		120	179.56	8.02	+
48		144	111.14	<1	+
49		168	61.15	<1	+
50	297627	I	0	<1	-
51		5	601	108	+
52		24	435	8.9	+

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2		48	61	<1	+
3		168	<1	<1	-
4	III	0	<1	<1	-
5		5	351	39	+
6		24	560	15	+
7		48	n.t.	n.t.	-
8		168	n.t.	n.t.	-
9	VI	0	73	1.4	+
10		5	1618	158	+
11		24	2761	110	+
12		48	319	7.9	+
13		72	87	1.7	+
14		96	60	1.2	+
15		120	n.t.	n.t.	+
16		144	44	<1	+
17		168	71	1.0	+

5 *n.t.: not tested

6 Table 3. Fluorescence data of REA test in groups D, A and C (CC α = 4409 Bovee *et al.*, 2008).

	Treatment day 15	Treatment day 44
Group D	1213 \pm 1714	-117 \pm 2569
Group A	3348 \pm 1223	1346 \pm 1340
Group C	376 \pm 330	1818 \pm 1860

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Table 4. The 2x2 contingency table for the evaluation of REA validity as a qualitative screening test (GC-MS method considered the golden test).

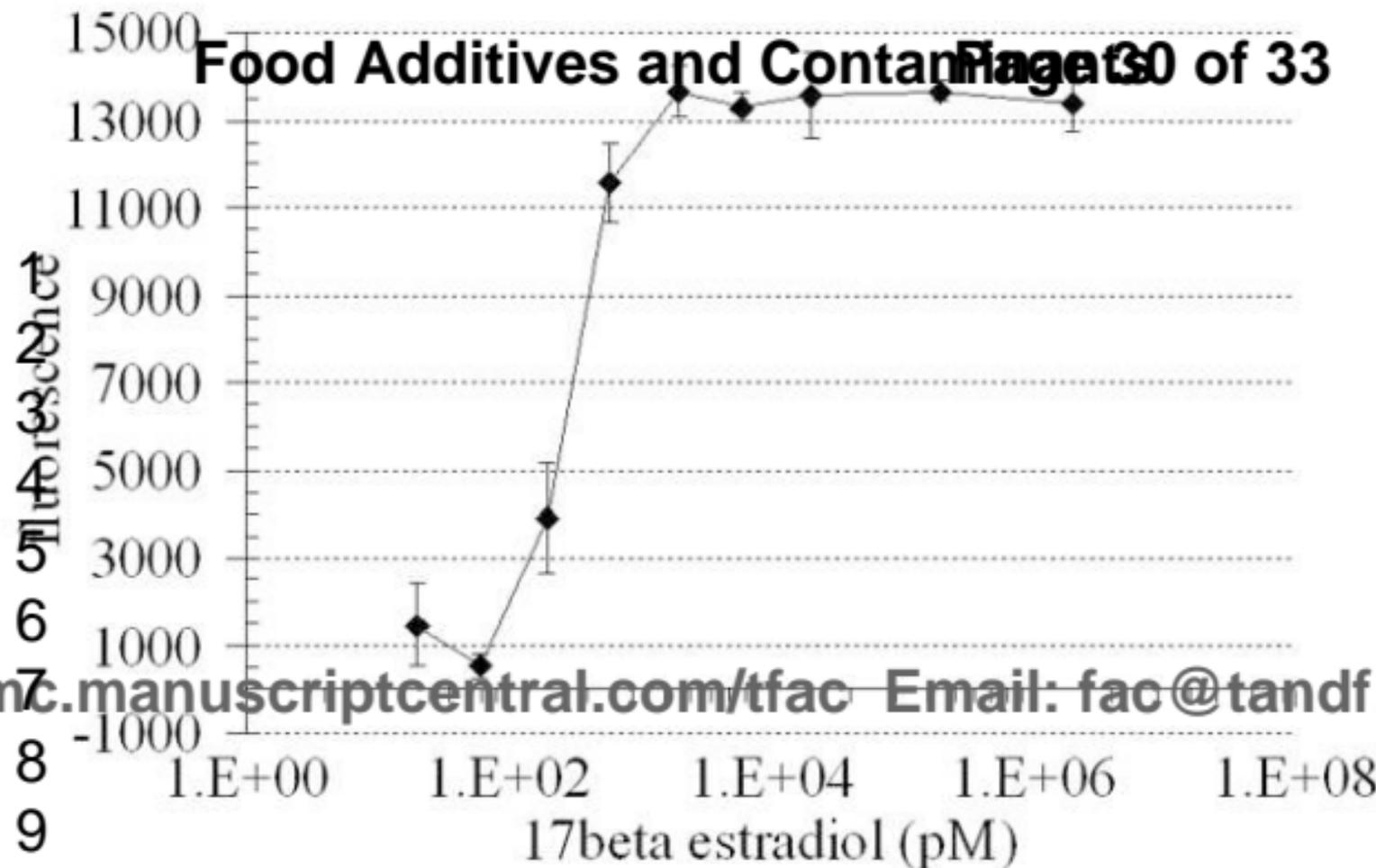
REA results	GC-MS estrogens positive	GC-MS estrogens negative
+	83 ^a	0 ^b
-	5 ^c	16 ^d

Deleted: 40
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^anumber of true positives
^bnumber of false positives
^cnumber of false negatives
^dnumber of true negatives

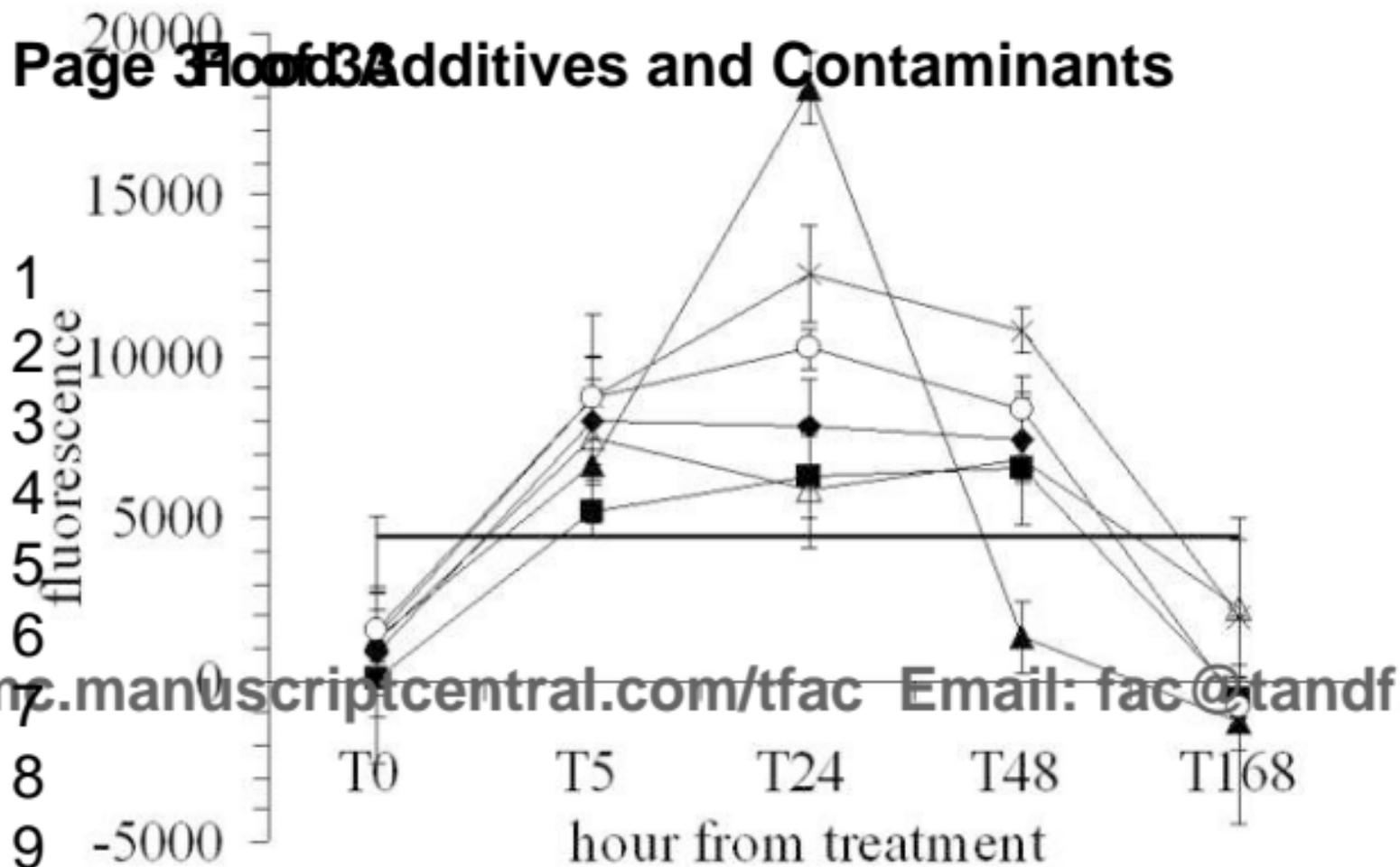
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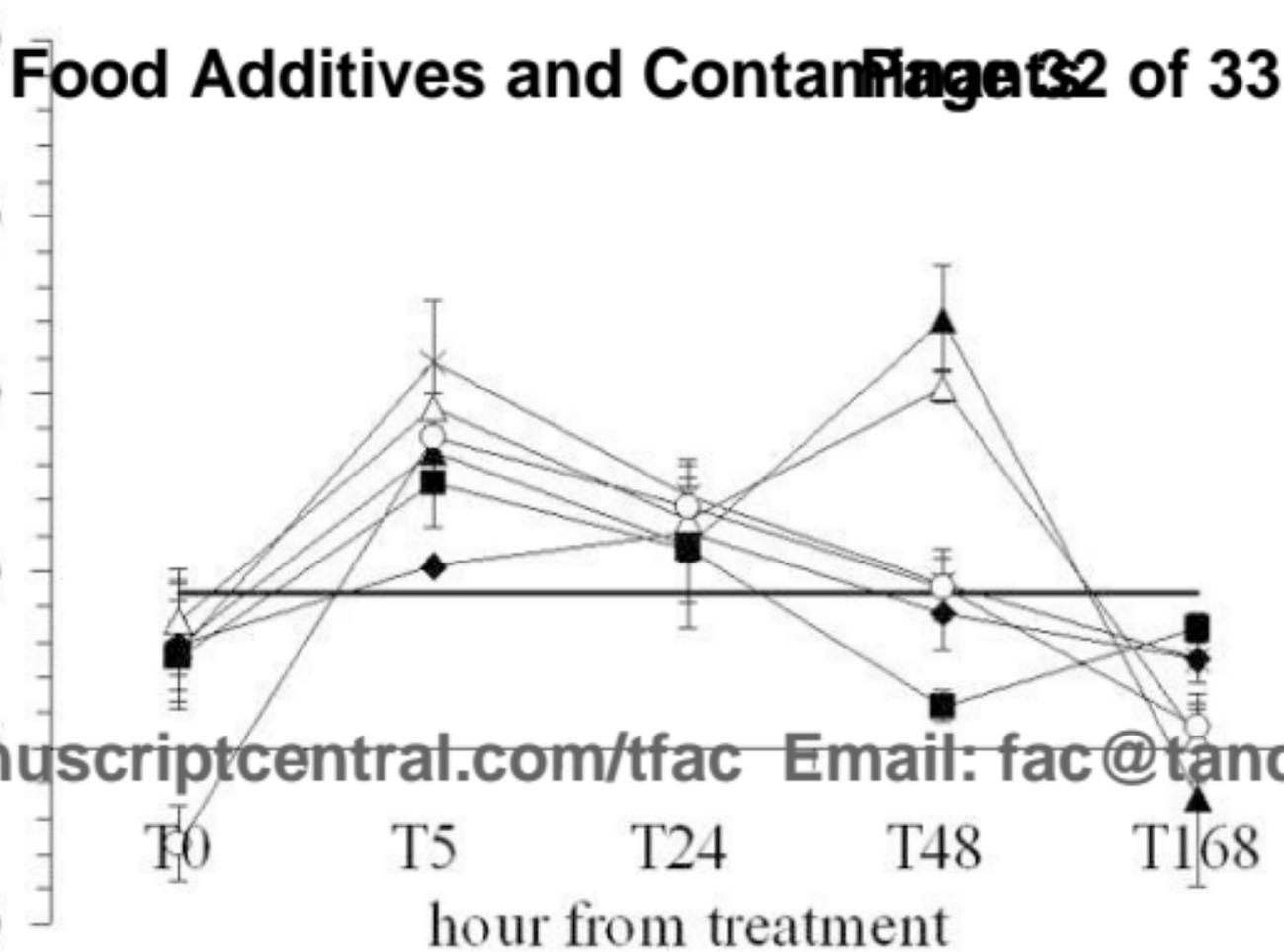


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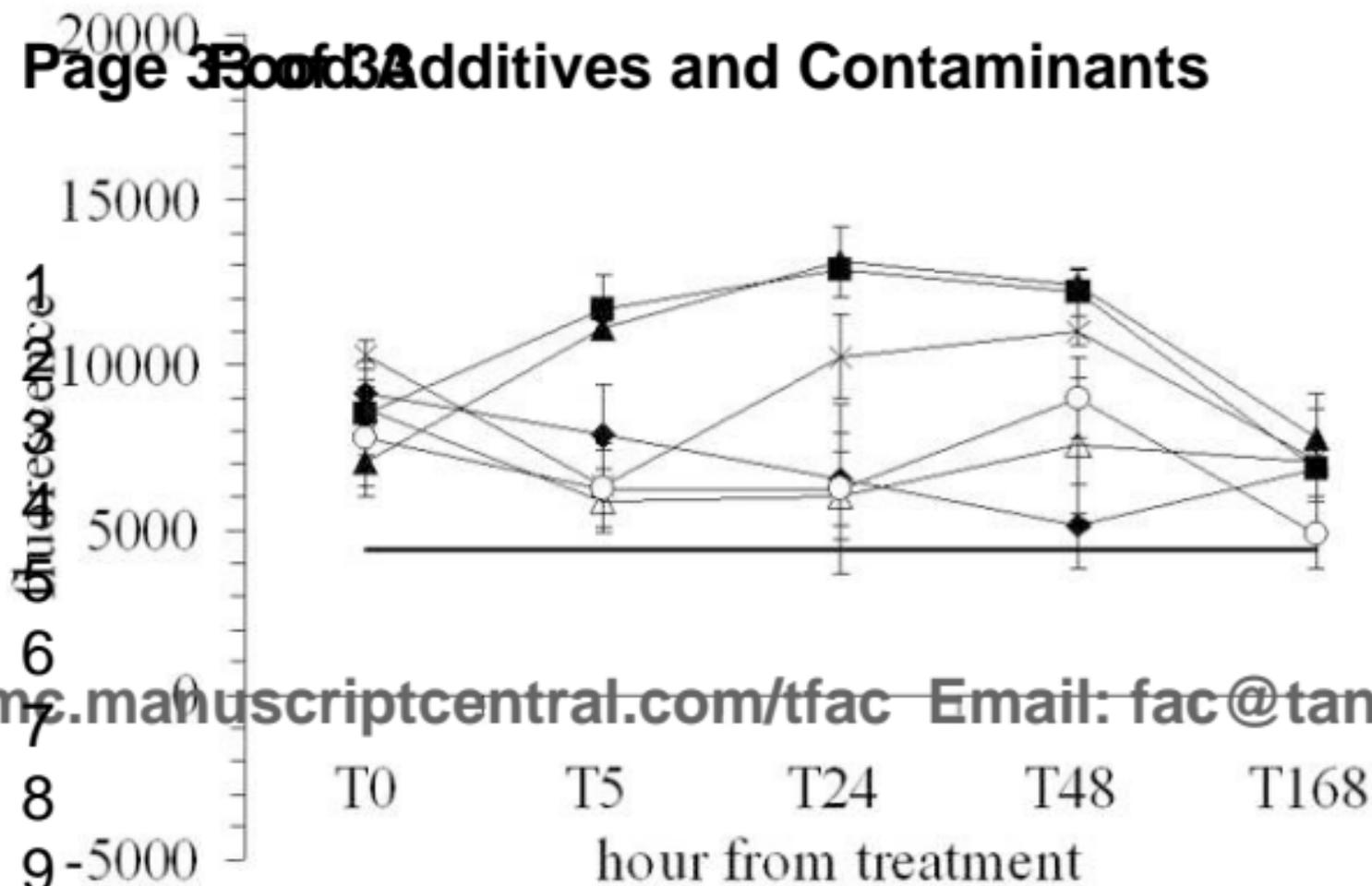
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