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## Estrogenic activity of estradiol and its metabolites in the ER-CALUX assay with human T47D breast cells

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A number of metabolites of 17 $\beta$ -estradiol were tested for their estrogenic activity using the ER-CALUX assay based on the increased expression of luciferase in exposed T47D breast cancer cells. E<sub>2</sub> $\beta$  and estrone showed similar potencies in the test, whereas E<sub>2</sub> $\alpha$  was 100 times less active. Incubation of cells with estrone (0.35  $\mu$ M) resulted in the formation of E<sub>2</sub> $\beta$ , whereas the reverse reaction was observed for E<sub>2</sub> $\beta$ . The resulting equilibrium may explain the similar estrogenic potency of estrone in the test. The synthetic 17-hydroxy benzoate ester of E<sub>2</sub> $\beta$  was 3 times less active than the parent compound. The 17-hydroxy palmitate and oleate esters of E<sub>2</sub> $\beta$ , were respectively 25 and 200 times less active than the parent compound. The 2-hydroxy metabolites of E<sub>2</sub> $\beta$  and estrone showed a 5,000 to 10,000 fold lower activity. The 4-hydroxy metabolites were more potent than the 2-hydroxy metabolites, showing only a 20–200 times lower activity. The 2- and 4-methoxyesters of estrone were 700 times less active. It is concluded that the estrogenic potency of metabolites formed in cattle after treatment with E<sub>2</sub> $\beta$ , like estrone, E<sub>2</sub> $\alpha$  and especially the esters of E<sub>2</sub> $\beta$ , may be significant with respect to the potential risk of the use of estradiol for growth promotion in domestic animals in certain countries.

Key words: Estrogenicity; ER-CALUX; catecholestrogens; estradiol; estradiol-esters.

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The use of 17 $\beta$ -estradiol for growth-promoting purposes in cattle may result in the increased formation of residues of not only the parent compound but also its metabolites. In order to investigate the potential risk for the consumer, it is essential to obtain information on the identity, levels and biological properties of these compounds. In cattle the major metabolites of E<sub>2</sub> $\beta$  are estrone, the 17 $\alpha$ -congener and their glucuronide conjugates (1–3). Previous studies showed increased levels of the parent compound, and the two major bovine metabolites,

17 $\alpha$ -estradiol and estrone, in various tissues of implanted steers (2–4). In addition, a number of fatty acid esters have been identified which are very lipophilic and may as such accumulate in the tissues (4, 5). In humans, E<sub>2</sub> $\beta$  and estrone have been shown to be partly metabolized to their 2- and 4-hydroxy metabolites, which may be further metabolized to their methoxy-esters. Thus far the possible presence of these catechols and methoxy-esters in tissues of heifers or steers has not been investigated.

The aim of the present study was to investigate the estrogenic activity of a number of estradiol metabolites using the recently developed ER-CALUX assay (6). This test is based on the

increased expression of a luciferase reporter gene by human T47D breast cancer cells following exposure to estrogens. In vitro models like the E-screen and ER-CALUX assays have been shown to be valuable tools for investigating the estrogenic potency of individual compounds.

The present study shows that the natural esters of 17 $\beta$ -estradiol are potent estrogens and suggests that these should be taken into account as residues in meat. The estrogenic potential of metabolites that may be present as residues may be especially important when evaluating the potential risk for those consumers with the lowest 17 $\beta$ -estradiol production, i.e. prepubertal boys. The higher estrogenic potency of the 4-hydroxy as compared to the 2-hydroxy metabolites of estradiol and estrone may further contribute to the higher carcinogenic potency of these metabolites (7–10). More generally, it is shown that the metabolic potency of the cells used in the assay may play an important role in the relative estrogenic potency of the test compound.

## MATERIALS AND METHODS

### *Materials*

Standards of 2-hydroxy-17 $\beta$ -estradiol, 4-hydroxy-17 $\beta$ -estradiol, 2-hydroxy-estrone, 4-hydroxy-estrone, 2-methoxy-17 $\beta$ -estradiol, 4-methoxy-17 $\beta$ -estradiol, 2-methoxy-estrone and 4-methoxy-estrone were purchased from Steraloids (Wilton, NH), 17 $\beta$ -estradiol, 17 $\alpha$ -estradiol, estrone and the 3-benzoate ester of 17 $\beta$ -estradiol from Sigma (St. Louis, US). The oleate and palmitate esters of 17 $\beta$ -estradiol were synthesized by Dr Paris from the Laboratoire Xénobiotiques of INRA in Toulouse (France) and were shown to be free of 17 $\beta$ -estradiol and estrone by GC and LC/MS analysis. Furthermore, the identity was confirmed by <sup>1</sup>H-NMR performed at the RIKILT. Radiolabeled [<sup>14</sup>C]17 $\beta$ -estradiol was obtained from N.E.N. (spec.act. 1.96 GBq/mmol). Radiolabeled estrone was purified from an incubation of human Caco-2-cells with 17 $\beta$ -estradiol. Due to their possible instability, most compounds were freshly prepared just prior to the experiments. However, because of the limited amounts of palmitate and oleate esters available, these compounds were dissolved at once and the same preparation repeatedly used.

### *Estrogenicity assays*

The ER-CALUX assay was carried out by the Division of Toxicology at the University of Wageningen. Modified T47D cells, expressing luciferase in response to exposure to estrogens, were exposed to test

compounds for 24 h in 96-well plates, essentially as described by Legler *et al.* (6). Compounds were dissolved in DMSO and diluted in the medium. Following exposure, cells were lysed and the luciferase activity in the lysates was determined by flash kinetics using a microplate luminometer.

### *Metabolism studies*

Monolayer cultures of wild-type T47D-cells were incubated in 6-well multiwell plates (Costar, Badhoevedorp, The Netherlands) with 1 ml of medium containing 0.35  $\mu$ M radiolabelled estradiol or estrone for 4 and 24 h. The medium was subsequently removed and extracted twice with 2 ml diethylether. For identification of metabolites, cells were incubated in 100 mm dishes with 7.5 ml medium containing 10  $\mu$ M unlabelled 17 $\beta$ -estradiol or estrone.

### *HPLC-analysis*

Following evaporation of the diethylether, the extracts were redissolved in methanol/water 1/1 (v/v) and analysed on an HPLC system. Metabolites were separated on a LC-ABZ column (250 $\times$ 4.6 mm ID; Supelco), heated at 40 $^{\circ}$ C and using a 1 min 50/50 isocratic elution with water/methanol/acetic acid 900/100/8 (eluent A) and water/methanol/acetic acid 100/900/8 (eluent B) followed by a 15 min linear gradient to 75% eluent B. Following UV-detection (290 nm), the eluent was mixed with scintillation cocktail prior to on-line radioactivity detection (LB506C, Berthold, Germany).

### *GC/MS analysis*

GC/MS was used to check the purity of standards, in particular the presence or absence of 17 $\beta$ -estradiol and estrone, and to confirm the identity of metabolites. An absolute amount of 1  $\mu$ g standard was derivatized with TMCS (trimethylchlorosilane)/BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide)/pyridine 1/10/90 (v/v) and, after evaporation to dryness, redissolved in 10  $\mu$ l dry iso-octane. Samples were investigated by GC/MS in single ion monitoring, using a DB-1 column (30 m $\times$ 0.25 mm ID; J&W Scientific, Rancho Cordova, USA) with a film thickness of 0.25  $\mu$ m. The GC/MS system (Hewlett-Packard, Rockville, USA) consisted of a Model 5890 Series II gas chromatograph, an Engine Model 5989B mass spectrometer (electron-impact mode), a Model 7673-A autoinjector and a Chemstation. Injection temperature was 280 $^{\circ}$ C, oven temperature, programmed from 130 $^{\circ}$ C (held for 2 min) to 250 $^{\circ}$ C (held for 5 min) at 18.5 $^{\circ}$ C/min, then to 300 $^{\circ}$ C at 7.5 $^{\circ}$ C/min. The source was maintained at 250 $^{\circ}$ C, the quadrupole at 120 $^{\circ}$ C. Estradiol was e.g. identified by its retention times and the fragments with m/z 416, 285, 326 and 129. Detection limit was 100 pg, consistent with an impurity of less than 0.01%. For confirmation of metabolites, the diethylether extracts were evaporated, derivatized and analyzed in a similar way.

*Data evaluation*

Dose-response curves were fitted with the software package SlideWrite Plus 6.00 using a one-ligand curve-fit according to the formula:  $\text{response} = (\text{max.response}) \times [\text{agonist}] / (\text{EC}_{50} + [\text{agonist}])$ . Curves were used to determine the EC<sub>50</sub> values (dose giving 50% response). Based on the EC<sub>50</sub> value, the estrogenic potency expressed as the relative estradiol equivalency factor (EEF) was calculated for each compound as  $\text{EC}_{50_{\alpha}} / \text{EC}_{50_{\beta}}$ .

## RESULTS

The estrogenic activity of 17 $\beta$ -estradiol and its metabolites was studied in a number of different tests. Typical results of the tests are shown in Figs. 1 to 4. The calculated relative estradiol equivalency factors (EEF), indicative for the estrogenic potency, are summarized in Table 1. In all studies, 17 $\beta$ -estradiol was the most potent compound, showing EC<sub>50</sub> values between 2 and 30 pM. However, the activity of estrone was very similar to that of 17 $\beta$ -estradiol (Fig. 1) and this was confirmed in three independent studies (data not shown). The 17 $\alpha$ -congener was far less potent (150–300 $\times$ ), showing an EC<sub>50</sub> value between 300 and 1000 pM.

The four catecholestrogens were less potent than the parent compounds. However, in all

studies the 4-hydroxy metabolites were clearly more potent than the 2-hydroxy metabolites (Fig. 2, Table 1). The methoxyesters of estrone were only tested once, also showing a much lower activity than the parent compounds (Fig. 3, Table 1). The 2 and 4-methoxy-estrone metabolites were of similar potency as the corresponding hydroxy metabolites. Estradiol-benzoate was tested twice and showed a slightly lower activity (2–7 $\times$ ) than the parent compound. The palmitate and oleate esters also showed a clear estrogenic activity although to a lesser extent than the benzoate ester.

Since even slight impurities of estradiol or estrone may be responsible for the observed activities, all compounds were analyzed by GC/MS. None of the standards showed detectable levels of estrone. However, several compounds contained traces of 17 $\beta$ -estradiol (Table 2), whereas the 2- and 4-methoxyesters of estradiol showed levels of parent compound of respectively 2.2% and 1.1%. This was clearly reflected in the relatively high EEF values observed for these compounds being 0.4 and 0.02 for the 2- and 4-methoxy esters respectively (data not shown). For this reason these results were omitted from the data.

In order to investigate the metabolism of 17 $\beta$ -estradiol in these type of cells, wild type T47D

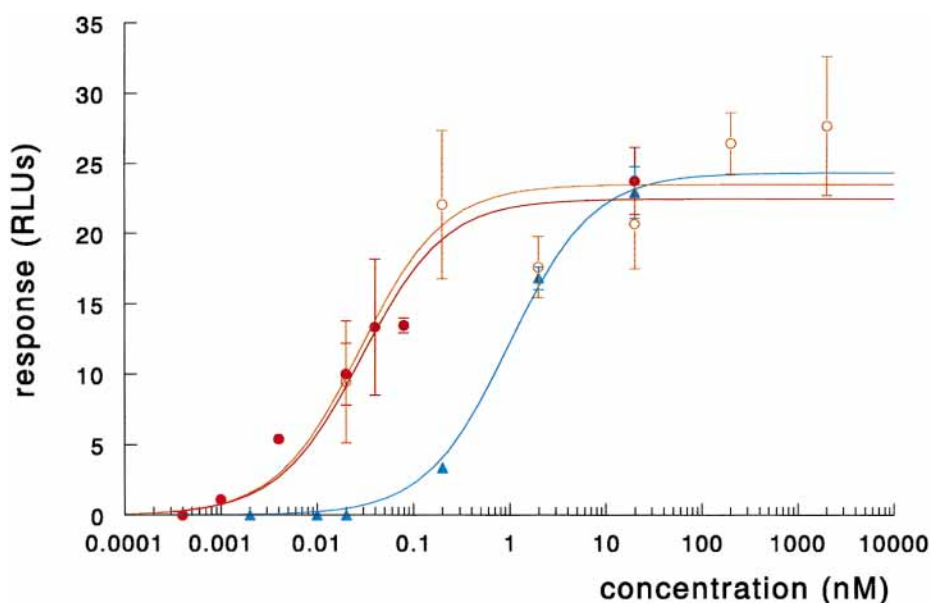


Fig. 1. Response of 17 $\beta$ -estradiol (closed circles), estrone (open circles), 17 $\alpha$ -estradiol (filled triangles) in the ER-CALUX assay. Results are expressed in relative light units (RLUs) as the mean  $\pm$  SEM ( $n=3$ ). The response obtained with the solvent control is subtracted. Data were fitted by 1-ligand curve fitting.

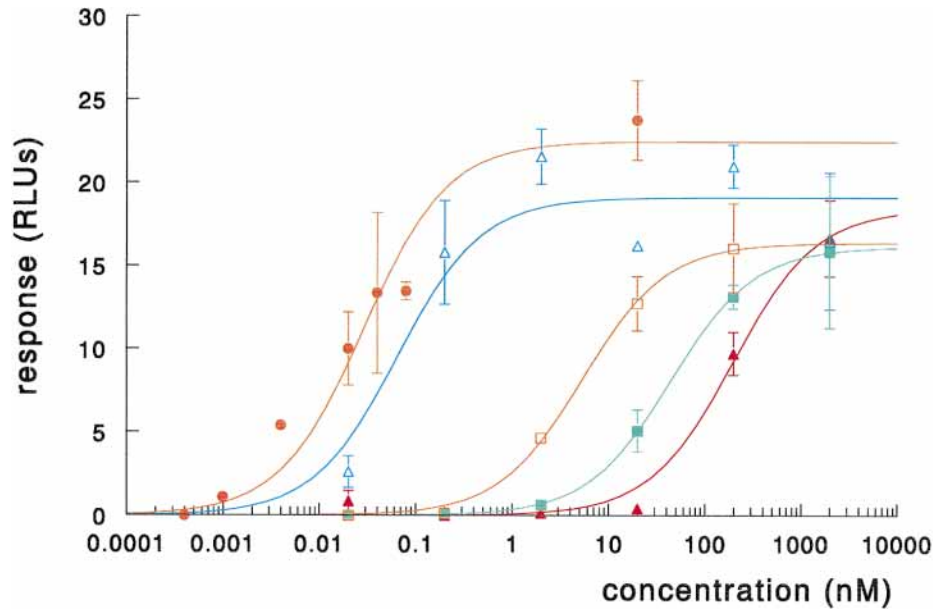


Fig. 2. Response of 17 $\beta$ -estradiol (closed circles), 4-hydroxy-17 $\beta$ -estradiol (open triangles) 4-hydroxy-17 $\beta$ -estrone (open squares), 2-hydroxy-17 $\beta$ -estrone (filled squares), and 2-hydroxy-17 $\beta$ -estradiol (filled triangles) in the ER-CALUX assay. Results are expressed as the mean  $\pm$  SEM (n=3). The response obtained with the solvent control is subtracted. Data were fitted by 1-ligand curve fitting.

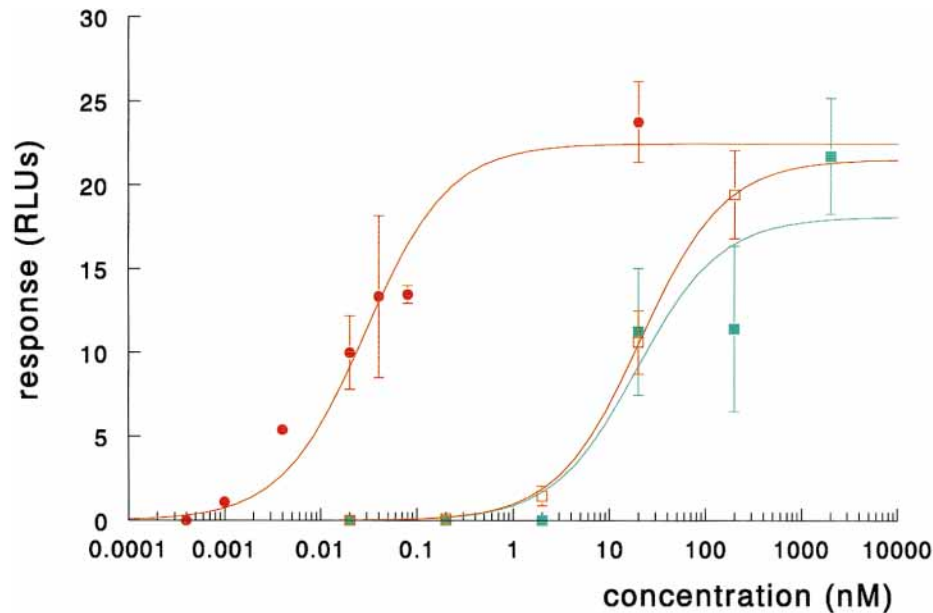


Fig. 3. Response of 17 $\beta$ -estradiol (filled circles), 4-methoxy-17 $\beta$ -estrone (open squares) and 2-methoxy-17 $\beta$ -estrone (filled squares) in the ER-CALUX assay. Results are expressed as the mean  $\pm$  SEM (n=3). The response obtained with the solvent control is subtracted. Data were fitted by 1-ligand curve fitting.

cells were incubated with radiolabeled estradiol. Treatment of media samples, taken after 4 and 24 hours, with diethylether resulted in the extraction of respectively 80% and 69% of the

radioactivity, whereas 8% and 27% remained in the water phase. Following washing of the cells with PBS, another 11% and 7% were released from the cells following a number of extractions



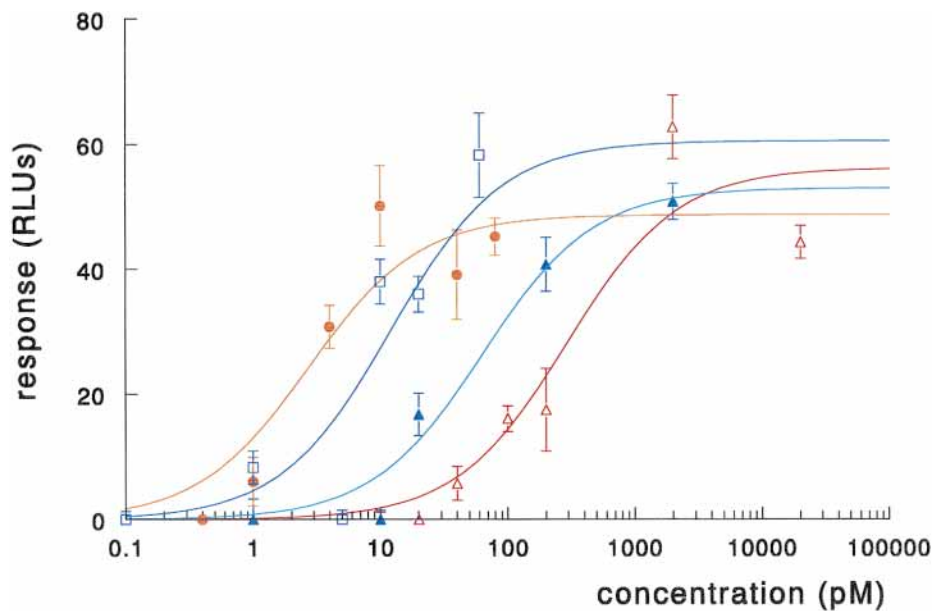


Fig. 4. Response of  $17\beta$ -estradiol (filled circles), the 17-hydroxy-benzoate (open squares), the 17-hydroxy-palmitate (filled triangles), and the 17-hydroxy-oleate esters (open triangles) in the ER-CALUX assay. Results are expressed as the mean  $\pm$  SEM ( $n=3$ ). The response obtained with the solvent control is subtracted. Data were fitted by 1-ligand curve fitting.

with PBS/methanol and methanol. After 24 h of incubation, the diethylether extracts of the media samples contained about equal amounts of the parent compound and estrone (Table 3). Incubation of cells with estrone resulted in the formation of a peak coeluting with  $17\beta$ -estradiol (Table 3). In this case 87% and 81% of the initial radioactivity was recovered in the diethylether extracts from media taken after respectively 4 and 24 h, with 3% and 15% remaining in the water phase. The cells were shown to contain

11% and 7% of the radioactivity. The formation of estrone from  $17\beta E_2$  and reverse was confirmed by GC/MS analysis of extracts from media taken from cells incubated with 10  $\mu$ M of the two compounds.

## DISCUSSION

Previous studies have shown that in vitro models, like the E-screen (11) and the ER-CALUX assay (6), are very suitable systems for investigating the estrogenic properties of compounds. A major advantage of mammalian

TABLE 1. Evaluation of estradiol equivalency factors (EEF) for the various metabolites

Compound	EEF
$17\beta$ -estradiol	1.0
Estrone	1.0
$17\alpha$ -estradiol	0.03
2-hydroxy- $17\beta$ -estradiol	0.00015
4-hydroxy- $17\beta$ -estradiol	0.45
2-hydroxyestrone	0.00065
4-hydroxyestrone	0.0054
2-methoxyestrone	0.0015
4-methoxyestrone	0.0014
$17\beta$ -estradiolbenzoate	0.25
$17\beta$ -estradiolpalmitate	0.05
$17\beta$ -estradiololeate	0.01

TABLE 2. Level of contamination of standards with  $17\beta$ -estradiol

Compound	Steraloids code	Level of impurity (%)
2-hydroxyestradiol	e2470	<0.01
4-hydroxyestradiol	e2500	0.03
2-hydroxyestrone	e1130	<0.01
4-hydroxyestrone	e1170	0.02
2-methoxyestradiol	e2490	2.7
4-methoxyestradiol	e2510	1.1
2-methoxyestrone	e1148	<0.01
4-methoxyestrone	e1175	0.02

No estrone was detected in these standards.

TABLE 3. *Metabolism of estradiol and estrone by T47D cells. Cells were incubated with 1  $\mu$ M of the radiolabelled compounds for 4 and 24 h. Results expressed as the fraction (%) of total radioactivity in the radiochromatogram*

Compound	Time (h)	17 $\beta$ -estradiol (%)	Estrone (%)
17 $\beta$ -estradiol	4	80 $\pm$ 4	14 $\pm$ 2
	24	59 $\pm$ 2	39 $\pm$ 1
Estrone	4	11 $\pm$ 1	85 $\pm$ 2
	24	43 $\pm$ 3	51 $\pm$ 2

cells, in comparison to modified yeasts, is their closer resemblance to the target cells *in vivo*, including the possible presence of mammalian enzymes involved in estrogen metabolism. The present study e.g., shows a higher estrogenic activity (EEF of 0.25) for the benzoate ester than observed previously in yeast (EEF 0.06;12). Esters, which have recently been shown to be important estradiol metabolites in cattle (4, 5), also showed clear estrogenic activity. As a result it seems likely that these esters also express this activity in consumers of meat containing these compounds. Regarding their lipophilic nature, such compounds may easily accumulate in the bovine tissues, but also be readily absorbed in the GI-tract and transported in lipid particles, initially circumventing degradation by the liver. Based on these considerations, it is expected that the relative estrogenic potency in treated animals or consumers may even be higher than observed in the present study. This is supported by a study of Odum *et al.* (12), showing very similar estrogenic potencies for 17 $\beta$ -estradiol and the 3-hydroxy-benzoate ester in the rat uterotropic assay.

Two other bovine metabolites, 17 $\alpha$ -estradiol and in particular estrone showed a marked estrogenic activity. The relative potencies were similar to the relatively potencies of 0.03 and 0.5 reported by Routhledge and Sumpter (13), using a yeast estrogen screen based on *S. cerevisiae* transfected with a gene encoding for the human estrogen receptor  $\alpha$ . However, the estrogenic potency of estrone was relatively high when compared to the activity observed in the E-screen, a proliferation assay with human MCF-7 breast cancer cells (11). In this proliferation assay with MCF-7 cells, estrone showed a very low estrogenic potency (EEF of 0.01), even

lower than the 17 $\alpha$ -congener (EEF of 0.1). These data were confirmed by Mäkelä *et al.* (14). However, these authors also investigated the conversion of estrone into 17 $\beta$ -estradiol by both MCF-7 and T47D cells and observed a 3–4 fold higher activity in the latter cells. This is in line with the effects observed in the present study, showing about equimolar concentrations of 17 $\beta$ -estradiol and estrone after 24 h of incubation (Table 3). It is expected that this equilibrium may be reached even earlier at the much lower concentrations needed to obtain an estrogenic response. The conversion of estrone to 17 $\beta$ -estradiol was also reported by Feldman and Krishnan (15) for *S. cerevisiae* yeast and may explain the similarity in the effects observed between the yeast assay and the ER-CALUX-assay.

The differences observed between the estrogenic potencies of the 4- and 2-hydroxy metabolites are of particular interest, also regarding reported differences in the carcinogenic potencies of these compounds (8–10). Recently, the higher estrogenic potency of the 4-hydroxy metabolite of estradiol was also reported in CD-1 mice (10), showing an increased uterine wet weight. Using a receptor binding assay with the human estrogen receptor, Van Aswegen *et al.* (16) and Kuiper *et al.* (17) also reported a higher affinity of the 4-hydroxy metabolite of 17 $\beta$ -estradiol as compared to the 2-hydroxy metabolite. According to Anstead *et al.* (18), this higher affinity may well be explained by the difference in the effect on the proton at the 3-hydroxy group which is essential for the binding to the receptor. However, the differences observed in the present study are much greater and this may again be explained by differences in the metabolism of the compounds. In the case of hamsters and rats, Li *et al.* (19) reported a higher activity of the enzyme COMT (catechol O-methyltransferase) for the 2-hydroxy metabolites, resulting in a faster deactivation of these metabolites as compared to the 4-hydroxy metabolites. Metabolic conversion, possibly in combination with their higher chemical stability in the culture medium, may also underly the relatively high estrogenic activity of the two methoxy-esters. Using a receptor binding assay the methoxy esters of 2- and 4-hydroxy estradiol showed very poor activity as compared to the hydroxy metabolites (16).

It is concluded that the natural esters should be taken into account when evaluating the total amount of estrogens present in meat, and in particular their increased levels in estradiol treated animals. The rather important metabolic capacity of the test organisms has previously been pointed out by Odum et al. (12). Although well known in the case of the esterase activity of the organisms used for in vitro estrogenicity screening, other possible differences in metabolizing enzymes may also contribute significantly to the relative potencies of specific compounds and should be taken into consideration.

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