Development, validation and routine application of the *in vitro* REA and DR-CALUX® reporter gene bioassays for the screening of estrogenic compounds and dioxins in food and feed

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Aan Ingeborg, Nick, Esmee en Lars

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General Introduction and outline of the thesis

Introduction

Food for animal or human consumption may contain residues of many different xenobiotics, such as environmental contaminants, pesticides, substances migrating from packaging materials, processing contaminants, veterinary drugs and growth-promoting agents, but may also contain natural toxins, like mycotoxins. For many of these compounds maximum residue limits (MRLs) have been established. Monitoring programs are required to check food items for the presence of residues. In particular in the case of potent bioactive compounds with low MRLs, or mixtures of compounds, such as in the case of polychlorinated aromatic hydrocarbons, such analytical methods are often laborious and expensive. As a result, monitoring can be performed on only a limited number of samples. The use of the newest generation of bioassays may overcome most of these problems and offer a number of additional advantages.

Since ancient history mankind has relied on bioassays to determine the safety of food and the environment. In medieval times, food tasters were employed to ensure that food was free of poisons. Miners used canaries to detect the possible presence of toxic gasses in the mining tunnels. With increasing knowledge about the responsible toxicants, improvements in analytical chemistry, combined with the need to reduce animal experiments, monitoring now relies on chemical methods aimed at the detection of compounds by their physical and chemical properties. The use of in vivo animal bioassays is more or less restricted to the testing for the presence of specific substances. For example, due to the absence of suitable analytical methods, even today in vivo bioassays with mice and rats are still the only reliable way to detect paralytic shellfish poison (PSP) and diarrheic shellfish poison (DSP) in shellfish[1,2] and the neurotoxins produced by Clostridium botulinum[3]. Fish assays are still widely used for testing the quality of drinking water and in the second Gulf War the US Army used small animals to detect toxins.

Despite the rapid improvements in analytical chemistry we start to realise that these methods may no longer be sufficient to deal with the often very complex mixtures of chemicals or ever changing chemical structures of toxicants present as residues in our food chain. Furthermore, there is a strong need for rapid screening assays that can be used for extensive monitoring programs. Bioassays with pro- or eukaryotic cells, capable of detecting compounds based on their effects, offer a possible solution. Bacteria are widely used to screen for the presence of antibiotics and antibacterial drugs in food[4,5], and in many cases chemical identification of the responsible substances is no longer required. Recent advances in cell biology and in particular biotechnology have allowed the development of a new generation of bioassays, based on the possibilities to introduce specific properties and reporter genes into stable cellular systems. The present thesis describes the development of such new generation bioassays and demonstrates their advantages, especially when used in combination with sensitive analytical methods. Thus, the thesis describes the development, validation and some examples of application of the so-called RIKILT yeast Estrogen bioAssay (REA), an in house developed bioassay used for the detection of estrogenic compounds and of the DR-CALUX® assay, a bioassay used for the detection of dioxins. Figure 1 shows the basic principle by which these receptor-based reporter gene assays work. An active compound or ligand (L) is recognised by a specific receptor (R). Following the binding of a ligand to its specific receptor, the ligand-receptor complex is transformed. For the estrogen receptor (ER) this is the dissociation of heat shock protein 90 (Hsp 90) that enables the occupied receptor to dimerise. For the aryl hydrocarbon receptor (AhR) this is the dissociation of two Hsp 90 molecules followed by the formation of a complex between the ligand occupied AhR and the AhR nuclear translocator (ARNT). The transformed ligand-receptor complex binds with high affinity to DNA at specific responsive elements (RE) and together with a complex of proteins and co-activators, this stimulates the transcription of an adjacent reporter gene by RNA polymerase II, resulting in the translation of a marker (M) that can easily be measured.

The inclusion of these new bioassays in modern test strategies will allow rapid screening and detection of both known and new, possibly unknown, agonists and help to evaluate the possible health hazards involved with the presence of such compounds in the food chain. In the case of the illegal abuse of hormones, this is of particular interest regarding the tendency to switch to novel compounds that thus escape from detection by chemical analytical methods. In the next sections the receptor-based reporter gene assays developed and optimised in the present thesis are introduced in some more detail.

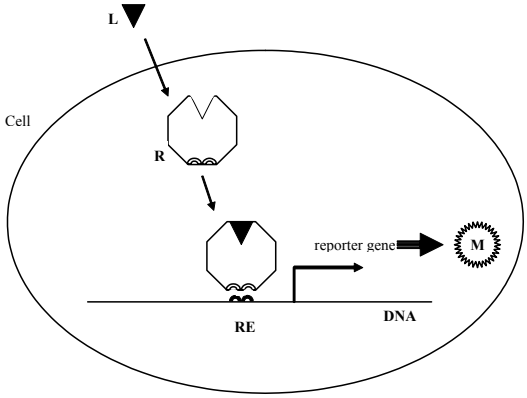

Figure 1. Schematic representation of the mechanism of action of the REA and DR-CALUX®. In case of the REA: the ligands (L) are estrogenic compounds like the female hormone 17β-estradiol, the receptor is the human estrogen receptor α or β (hERα or hERβ), the responsive elements are estrogen responsive elements (EREs) and the marker is yeast enhanced green fluorescent protein (yEGFP). In case of the DR-CALUX®: the ligands are aromatic hydrocarbons like dioxins and PCBs, the receptor is the aryl hydrocarbon receptor (AhR), the responsive elements are dioxin responsive elements (DREs) and the marker protein is luciferase (Luc).

1. Dioxins and the DR-CALUX® bioassay

Polychlorinated dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF) and biphenyls (PCB) are industrial compounds or by-products that have been widely identified as environmental contaminants. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic member of this class of halogenated aromatic hydrocarbons (HAH). Figure 2 shows the structure of these compounds. After the discovery of dioxins in the food chain, it became clear that it would be impossible to set up large monitoring programs for this group of compounds. The major reason for this was the expensive and laborious analytical procedure, required to detect 17 different 2,3,7,8-chlorinated dibenzo-p-dioxins (PCDDs) and furans (PCDFs) at the pg per g level. This can only be achieved after extensive clean-up and by using a high resolution mass spectrometer. A set of so-called toxic equivalency factors (WHO-TEFs), ranging from 1.0 to 0.0001, has been developed by the Word Health Organisation (WHO) in order to express the concentrations of each of the congeners into one unit, relative to the toxicity of the most toxic congener TCDD[6]. Thus the WHO-TEF value of a compound expresses its toxic potency in comparison to the most toxic congener TCDD (TEF of 1.0)[7,8]. The analytical determined levels of the different congeners are multiplied by this TEF value and subsequently summarised to obtain the total toxic or dioxin equivalent content (WHO-TEQ). These TEFs in combination with the TEQ principle were developed in order to deal with the mixtures of dioxins and PCBs, of which only a limited number are capable of binding to the Ah-receptor, each with their own affinity and resulting biological potential.

In the EU, typical tolerance limits for food are the maximum residue limits of 3 pg WHO-TEQ per g fat for dairy products, 2 pg WHO-TEQ per g fat for poultry meat and 1 pg WHO-TEQ per g fat for pork. The action levels, used to detect samples with levels above background but below the tolerance limit, are even lower and are in general 60% of the tolerance limit. Levels like this are required to meet the tolerable weekly intake of 14 pg WHO-TEQ per kg body weight as set by the Scientific Committee of Food (SCF). It is clear that many other substances like the planar non-ortho and mono-ortho PCBs and most likely also some of the brominated polyaromatic hydrocarbons should be included in these limits based on their dioxin-like effects and persistence. In 2006 the 12 dioxin-like PCBs will actually be included in the EU-limits.

In response to the limited analytical capacity, bioassays with mammalian cells have been developed, initially based on the known effects of these compounds. Following the binding of dioxins to the soluble cellular aryl hydrocarbon receptor (Ah-receptor), the ligand-receptor complex is transformed and subsequently translocated to the nucleus, where it binds with high affinity to DNA at specific so-called dioxin responsive elements (DREs), stimulating transcription of adjacent genes[9,10]. Normally this results in an increased transcription of genes coding for enzymes, such as the

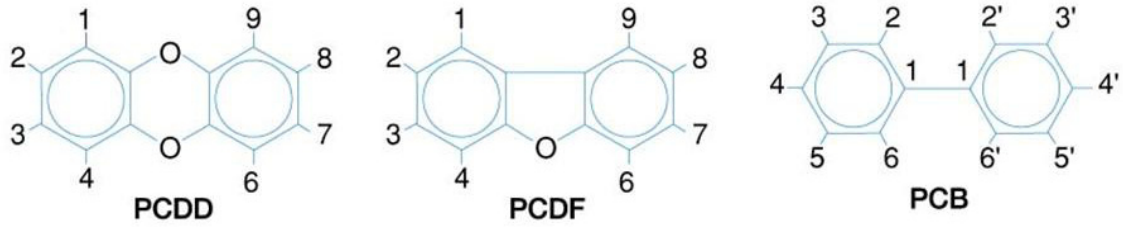

Figure 2. General molecular structure of polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCB).

cytochrome P450 IA subfamily and UDP-glucuronyl transferase. The cytochrome P450 IA dependent O-deethylation of ethoxy-resorufin (EROD) is often used as an in vivo or in vitro biomarker for exposure and in particular for the determination of dioxin levels in various samples like sewage sludge and sediments[11]. A major drawback of this system is the possible inhibition of the enzyme by many different compounds, including natural occurring substances. The specificity of the assay for dioxin detection was therefore tremendously increased by the development of cell-lines that contain the reporter gene luciferase under control of DREs isolated from the 5'-flanking region of the mouse P450 IA1 gene[12,13]. In response to dioxins, these rat and mouse hepatoma cell-lines will synthesise luciferase in a dose-dependent way, which can subsequently be quantified by an enzymatic light producing reaction[14]. Figure 3 shows a schematic representation of the mechanism of action of this new H4IIE rat cell-line (DR-CALUX® bioassay) and Figure 4 presents a typical dose-response curve, showing an increased luciferase activity at concentrations as low as 0.5 pM TCDD. For comparison Figure 4 also depicts the TCDD dependent induction of EROD activity in the H4IIE cells. In principle, the amount of dioxins in a sample can be quantified by comparison of the response in the test with the calibration curve for TCDD and is then expressed in TCDD equivalents. However, this is only valid if the cell line obeys the TEF/TEQ principle.

Several other dioxin and PCB congeners have been tested and were shown to give a response which reflects the differences in their WHO-TEF values. In general the DR-CALUX® bioassay obeys the TEQ-principle, showing additivity of the individual responses of the congeners (Chapter 2). This was expected as most toxic and biochemical effects of dioxins and PCBs are mediated by their ability to bind to the AhR[15].

False negative results

A disadvantage of the EROD-assay is the fact that cytochrome P450 IA can be irreversibly inhibited by a number of compounds[16]. Exposure of cells to an agonist in the presence of such an inhibitor would result in a false-negative test result. A known class of cytochrome P450 IA inhibitors are the benzimidazole drugs, used as fungicides, anthelmintic drugs and anti-gastrics[17]. These compounds showed negative results in the EROD-assay with H4IIE-cells. Figure 5 shows the effect of the anthelmintic benzimidazole drug oxfendazole on the TCDD induced EROD (A) and luciferase (B) activity. At relatively low doses, oxfendazole completely inhibits the EROD-activity (A), whereas in the case of the DR-CALUX® assay there appears to be an additive rather than an inhibitory effect (B)[17]. This confirms the insensitivity of the DR-CALUX® assay for these kinds of false-negative results and thus far no inhibitors for the luciferase enzyme have been reported. Several compounds have been reported as potent antagonists of the Ah-receptor pathway and such compounds might in theory cause false-negative effects when they are present in the test sample together with the agonist under investigation. On the other hand such compounds could be used to investigate whether an increased signal was caused by a real Ah-receptor agonist. Resveratrol, α-naphtoflavone and 4-amino-3-methoxyflavone were tested for their antagonism, but failed to show the expected effect in the DR-CALUX®. The latter two compounds actually caused a positive effect themselves[17]. A clear non-competitive Ah-receptor blocker remains to be identified. However, these three known antagonists failed to antagonise the effect of the strongest Ah receptor agonist, 2,3,7,8-TCDD, but might be able to antagonise the effect of less potent compounds like 1,2,3,4,7,8-HxCDD and PCB 126 (both a WHO-TEF of 0.1). At present the only clear cause of false-negative effects may be compounds causing cytotoxic effects and thus preventing the cells from responding to the agonists. However, in general cytotoxic effects are clearly recognised by visual inspection of the cells after the exposure and a decreased response in comparison to the control.

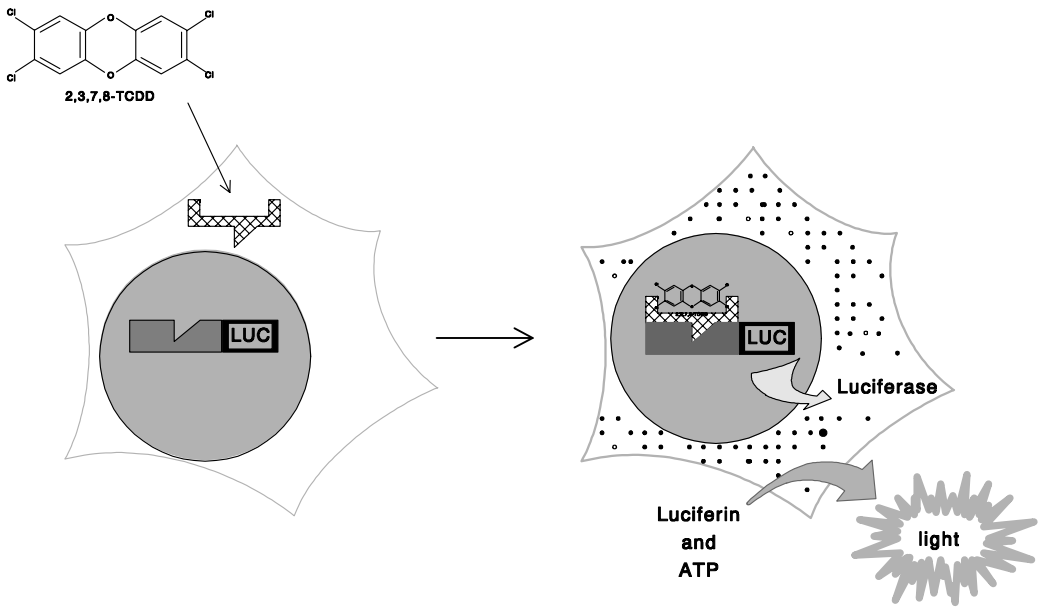

Figure 3. Schematic representation of the mechanism of action of the DR-CALUX®.

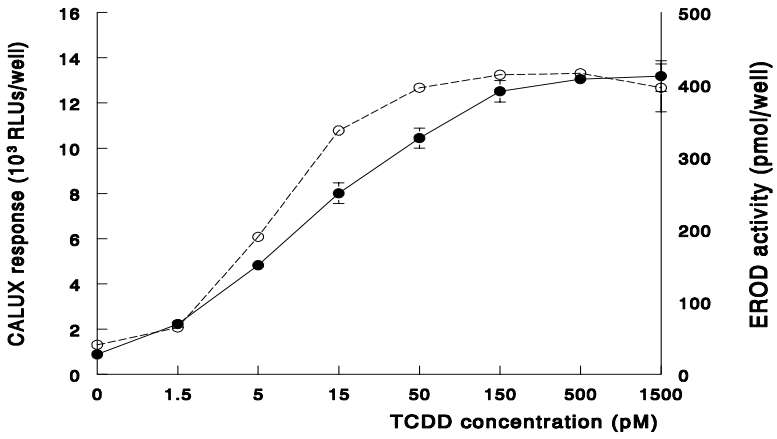

Figure 4. Dose-response curves for TCDD in the DR-CALUX®: Luc (•) and EROD (o) assays[17].

False-positive results when screening for dioxins and PCBs only

In addition to a number of dioxin and PCB congeners and their brominated analogues[18], the assay has been shown to be sensitive to a number of polyaromatic hydrocarbons (PAHs)[19]. As a result, the assay was shown to be suitable for the rapid screening of coconut oil for the presence of these types of PAHs[20]. However, as shown in Figure 6, low concentrations of benzo(a)pyrene (B(a)P) are only detected after a relatively short incubation time of 4 hrs. Following exposure to high concentrations of B(a)P, a clear response is also observed after 20 hrs. This relatively low level of luciferase induction upon prolonged incubation with low concentrations, is thought to be due to both the metabolism of B(a)P by the cells, as well as the instability of the luciferase formed during the first hours of exposure. This is supported by the results obtained with the newly developed CAFLUX assay, expressing the more stable enhanced green fluorescent protein instead of luciferase[21] and showing high levels of reporter protein expression even upon prolonged exposure to low concentrations of B(a)P. Based on actual concentrations in samples, this feature allows a discrimination between the effects of

PAHs and dioxins, by varying the incubation interval. Also the well known Ah-receptor agonists α- and β-naphtoflavone were shown to give a response in the DR-CALUX® test[17]. Both examples, the PAHs and α- and β-naphtoflavone, are no real false-positives, but clearly show that the test is not only sensitive for dioxins and PCBs.

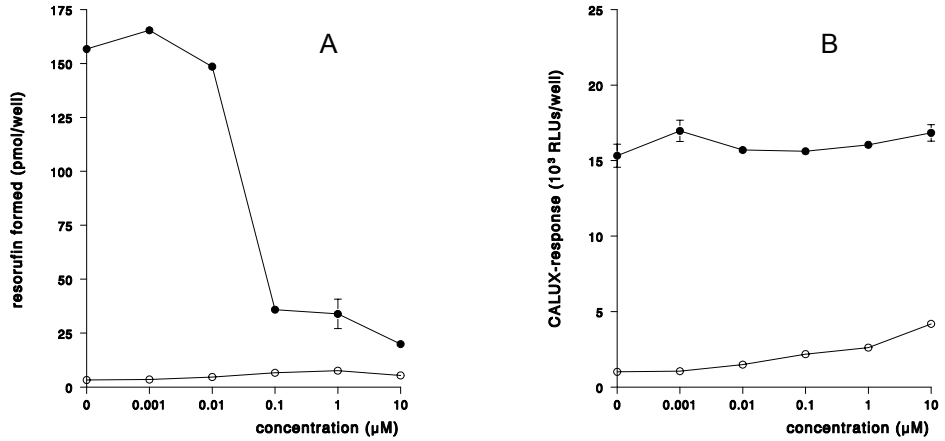

Figure 5. Effect of oxfendazole alone (o) or in combination with 100 pM TCDD (•) in the EROD (A) and DR-CALUX® (B) assay[17].

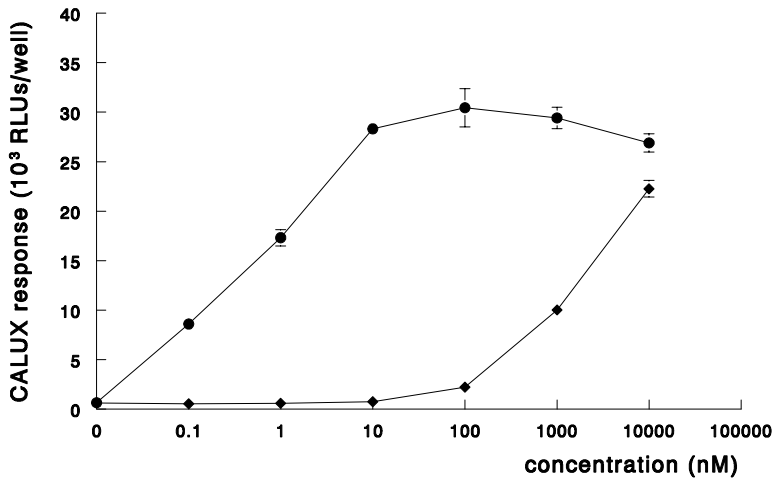

Figure 6. Effect of benzo(a)pyrene in the DR-CALUX® assay, following exposure of cells for 4 (•) and 20 hours (♦)[17,20].

As shown in Figure 5, a slight positive effect was also observed with oxfendazole, belonging to the group of the benzimidazoles. Similar effects were observed with other members of this group like febantel, fenbendazole, thiabendazole, mebendazole, lanzoprazole, omeprazole and benomyl[17]. Incubation with 1 mM thiabendazole resulted in a response (18406 ± 2218 RLUs/well) similar to the plateau value obtained with 500 pM TCDD (17714 ± 836 RLUs/well). However, although it is still questionable whether benzimidazoles are real Ah receptor agonists[22,23], based on current regulations, these compounds should not be present in food derived from food-producing animals at levels that could induce a positive effect.

A third group of compounds, showing a slight positive effect in the assay, are the corticosteroids, like hydrocortisone, corticosterone and dexamethasone. These compounds alone show a small and dose-related positive effect, but can also dramatically enhance the effect of TCDD. In the latter case the EC_{50} value shifts from 16 pM for the cells exposed to TCDD only to 8, 6 and 4 pM for cells exposed to TCDD in the presence of respectively 100 nM corticosterone, 100 nM hydrocortisone or 10 nM dexamethasone[17]. It is very likely that these compounds are present in certain type of samples, in particular blood plasma, resulting in a false-positive result or an overestimation of levels of Ah-receptor agonists. Thus, the reason for the increased response observed when cells were exposed to only corticosteroids, is still unclear. It is probably caused by the presence of the glucocorticoid responsive elements (GREs) in the mouse mammary tumor virus (MMTV) promoter that was used to introduce the DREs in the DR-CALUX®. However, the GREs might even not be necessary for the glucocorticoid receptor (GR) to induce transcription activation at the MMTV promoter. Stavreva and McNally showed a model where histone H1 phosphorylation and chromatin remodelling play key roles in regulating GR exchange at MMTV, and where these processes ultimately impact transcription from MMTV[24], but it is not clear whether the authors are aware of the GREs in the MMTV sequence. There are also indications that the effects of the corticosteroids are due to an indirect effect of these compounds on the Ah-receptor pathway, probably resulting in an increase in the concentration of the receptor, since the effects also occur with EROD-activity[25]. However, altogether the mechanisms involved are far from clear[26], but it is questionable whether all authors were aware of the GREs that are also present in the promoter sequence of the cytochrome P450IA1 gene[27,28].

In general, it will be questionable whether a positive result is really false or simply due to a possibly unknown agonist, which has been overlooked by more specific chemical-analytical methods. As such, these assays are extremely suitable for the detection of unknown or new agonists with potential health risks for the consumer. On the other hand, current regulations are directed towards specific compounds or classes of compounds and not towards compounds with a certain biological effect. Furthermore, the bioassay-response obtained from unknown or new agonists does not take into account factors like absorption, distribution, metabolism and excretion of compounds, which to a large extent are responsible for the toxicity of the accumulating and persistent dioxins. For this reason it is questionable whether a response in the test should be expressed in dioxin levels, or whether a sample should be regarded as suspected rather than positive when showing an elevated response in the test. Furthermore, besides the use of long exposure periods to improve the specificity of the test, e.g. in the case of benzo(a)pyrene the cells were able to metabolise both the active compound and the luciferase produced during the first hours of incubation, the use of a simple but selective clean-up procedure is necessary to make the assay more specific for the PCDD/F and PCB class of agonists.

The aim of the research described in this thesis was to make the DR-CALUX® applicable for the screening of dioxins and dioxin-like PCBs in food and feed, to validate the assay and to demonstrate its applicability in routine monitoring and during incidents. In Chapter 2, the TEFs of the most important and potent PCDD/Fs and PCBs were determined and a selective clean-up procedure was developed. Furthermore, the chapter describes the first validation study for food, more in particular, bovine milk fat, which was essential for the routine application of the test in The Netherlands. Subsequently, the bioassay was used successfully by RIKILT in at least five short-term incidents and a number of more structural issues like dioxin pollution in eel from polluted rivers and in eggs from free-ranging chickens. In Chapter 5, the applicability the DR-CALUX®-assay is demonstrated with animal feed samples from the German bakery waste incident and with fat from slaughtered pigs that were fed with the contaminated feed.

2. Estrogens and the RIKILT yeast Estrogen bioAssay (REA)

Estrogens

Hormones are compounds produced in specialised tissues and are subsequently transported via the blood stream to their effector sites. Steroid hormones represent a subgroup mediating their action via a large group of related proteins, the super family of nuclear receptors (NRs). All steroids are synthesised from cholesterol (C27) (Figure 7). Carbon atoms are removed, subgroups are added and ring structures changed. Steroid hormones in this pathway include progesterone (C21), testosterone (C19) and estradiol (C18). Obviously, the pathway provides one way of interaction between different compounds and different steroid receptors.

Members of the nuclear receptor group include, among others, the estrogen, androgen, progesterone, thyroid, vitamin D and retinoic acid receptor. Furthermore, a number of orphan receptors have been identified of which ligands and functions are largely unknown. Estrogens are C18-steroids that are made primarily in the female ovaries, are found in greater amounts in females than males and are thus often referred to as the female hormones. Estrogens influence the growth, differentiation and functions of many target organs, such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate. Estrogens also play an important role in bone maintenance, the central nervous system and in the cardiovascular system. Most effects of estrogens are mediated by estrogen receptors (ER) alpha and beta. The ERα is mainly expressed in the sex organs like the mammary gland and the uterus. ERβ is very important in the bone, urogenital tract, cardiovascular system, central nervous system and the developing brain. After binding of a ligand to the ER, dissociation of heat shock protein 90 (Hsp 90) enables occupied ERs to dimerise. The resulting dimer complex exhibits high affinity for specific DNA sequences, referred to as estrogen responsive elements (EREs), located in the 5' regulatory region of estrogen inducible genes. The ligand occupied ER-dimer functions as a transcription factor that modulates the activity of these responsive genes[29]. Gene transcription can be modified by cellular coactivators, repressors and modulators. Following transcription, mRNA is translated into protein by ribosomes. By inducing the synthesis of new proteins that alter cellular functions, estrogens have effects on cell function and physiology[30].

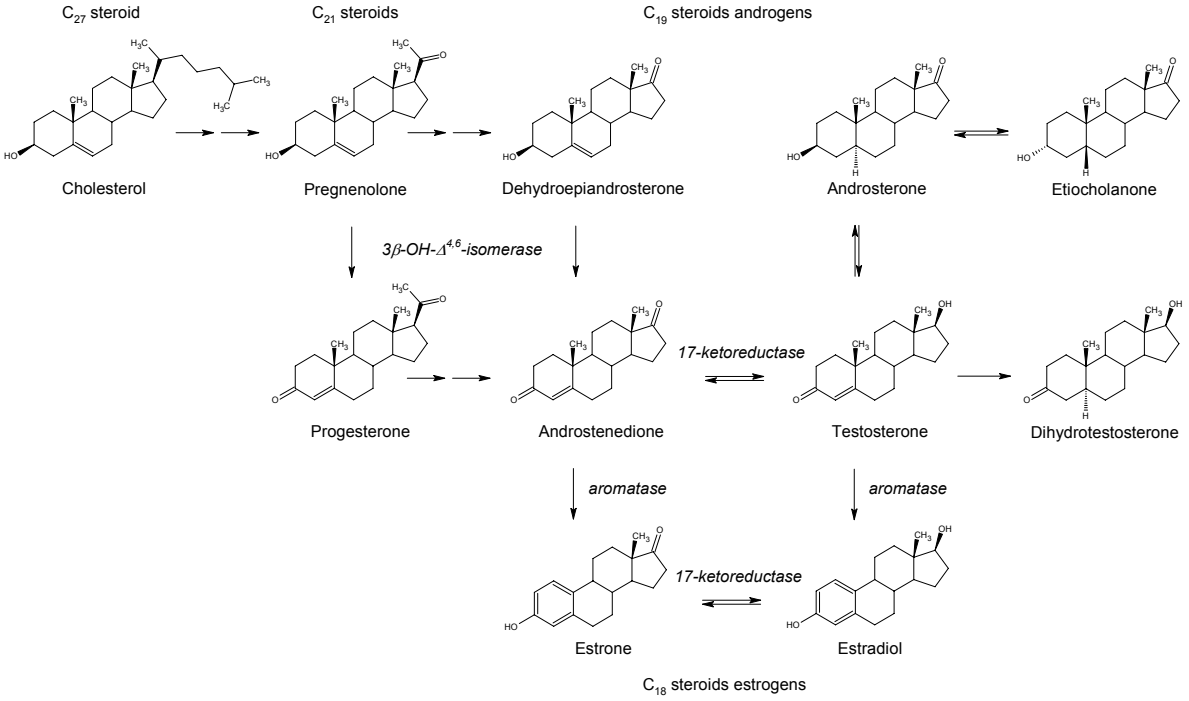

Figure 7. General molecular structure of steroids.

During the past decades, a large number of structurally diverse chemicals have been released into the environment. Many of these chemicals and waste products have steroid hormone-like activity and accumulate in the air, water and food chain. By acting as estrogen mimics (xenoestogens), they may disrupt normal endocrine function, possibly leading to reproductive failure in humans and wildlife and tumours in estrogen sensitive tissues[31,32,33]. This relates to chemicals with previously unknown hormonal properties, like certain pesticides and plasticizers, but also compounds used in the female birth control pill, tablets for hormone replacement therapy, the endogenous steroids excreted in urine of man and domestic animals and potentially also compounds used for their growth-promoting properties in animals. Analytical-chemical and immunological methods are commonly used to detect steroid hormones in food and feed, clinical practice, environmental samples or doping control. Due to the great variety of chemicals with hormone-like activity, these methods have the drawback that they only quantify the compound of interest and are not able to determine biological activity of unknown compounds and their metabolites, this in contrast to biological assays. Animal in vivo studies such as

the Allen-Doisy and Hershberger tests are used for the assessment of estrogenic and androgenic potencies respectively[34,35]. These tests are highly valuable to assess the overall biological effect of a compound. However, due to high costs, labour intensiveness, relatively poor sensitivity and modest responsiveness, in vivo assays are generally unsuitable for large-scale screening. Similarly, histopathological effects in tissues can be examined and used to establish the use of growth promoting agents in slaughtered animals[36]. However, these tests are only useful at the end of the food production, as they can not be used to test animal feed, illegal preparations or urine. Furthermore, they can detect the use of compounds but not the compounds themselves which may have been eliminated before the animals are slaughtered.

In vitro assays, such as competitive ligand binding assays and cell proliferation tests are more suitable for these screening purposes. Competitive ligand binding assays are rapid and easy to perform, but they determine the binding to the receptor and thus cannot distinguish between receptor agonists and antagonists. Cell proliferation assays, such as the E-screen[37], use ER-positive, estrogen-responsive MCF-7 (E-screen) or T47-D human breast cancer cells which show increased proliferation upon exposure to estrogen agonists. However, MCF-7 cells also express androgen, progesterone, glucocorticoid and retinoid receptors. This may compromise the suitability of the assay if extracts also contain substances that are able to bind to other receptors, as it has been shown that androgens, progestins and glucocorticoids can antagonise E2-induced cell proliferation. Furthermore, proliferative responses occur only after a number of days[38,39,40], resulting in a test that is not very fast. Alternatively, receptor based transcription activation assays can be used to detect all compounds having affinity for a given receptor[41,42]. In contrast to receptor binding assays, receptor gene bioassays also include the transactivation step and can distinguish between receptor agonists and receptor antagonists[42,43]. 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. Several reporter gene assays have been developed for this purpose, using both mammalian and yeast cells.

Mammalian cell lines

There are several transfected mammalian cell lines used to determine the estrogenic properties of compounds and sample extracts, mainly water and sediment. Most of these transfected cell lines are human and contain luciferase as the marker protein. In general it is thought that recombinant reporter gene assays based on stably transfected cell lines provide one of the most specific, sensitive and biologically relevant means to screen substances for their estrogenic effects. With regard to the sensitivity, human cell line based assays have been shown to be more sensitive than e.g. yeast based assays[44,45]. With regard to specificity and biological relevance, human cell lines may be able to identify compounds that require metabolism for activation into their active state[43,46,47]. However, the latter is not necessarily an advantage of the cell lines, since it does not have to reflect the effects observed in animals. This is demonstrated by cell lines that contain the aromatase enzyme, which converts androgens into estrogens[48]. Another example is the ER-CALUX assay that is based on the T47 D human breast carcinoma cell line. In this test, when compared to 17β-estradiol (E2), the relative estrogenic potency (REP) of estrone (E1) was 0.2 and that of estriol (E3) was 1.0[49]. The high potency of E3 could not be explained. In another study with this ER-CALUX cell line it was demonstrated that estrone had equal potency as E2 and a REP of 1.0 instead of 0.2 was found. In the latter study this could be explained by the fact that estrone was converted to 17β-estradiol and vice verse[47]. Another important issue is the effect of so-called anti-estrogenic compounds in the reporter gene assays. A further comparison of the performance of the ER-CALUX cell line with the performance of a recombinant yeast screen (YES) provided by Glaxo Group Research Ltd, United Kingdom was made[50]. In that study the estrogenic and anti-estrogenic properties of ICI 182,780, tamoxifen and 4-hydroxytamoxifen (OHT) were investigated. In the ER-CALUX test all three “anti-estrogens” completely abolished the luciferase induction by E2, while in the yeast test none of the “anti-estrogens” tested could antagonise the β-galactosidase activity induced by E2. In the ER-CALUX assay only tamoxifen was a very weak inducer at high concentrations (10% of the maximum response), whereas all three compounds showed a clear response in the yeast assay (30% of the maximum response and much less than suggested in table 1 of that paper; Legner, personal communication). Because of the differences in sensitivity to estrogens and anti-estrogens, further studies by this group were only carried out with the ER-CALUX assay. However, the authors were assuming that all three compounds were pure anti-estrogens, giving no response in the ER-CALUX and over 30% in the recombinant yeast test. Recent studies show that both tamoxifen and OHT are not pure anti-estrogenic compounds, but Selective Estrogen Receptor Modulators (SERM). Tamoxifen and OHT are often anti-estrogenic in breast, but are full agonists in the uterus. Tamoxifen was used to treat breast cancer patients because of its anti-estrogenic properties in breast, but was recently

replaced by raloxifen because tamoxifen promotes uterine cancer, due to its strong estrogenic effect in the uterus. Thus the 30% activity of tamoxifen and OHT in the yeast assay is not a false-positive result as is the lack to show anti-estrogenic activities. In fact it corresponds better to the in vivo situation in humans than effects observed with the human breast cancer cells. Unfortunately, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) adopted the conclusion and assumed that both tamoxifen and OHT are pure antagonists. This was one of their reasons to recommend the mammalian cell lines above the yeast assays[51].

Yeast cells

Until now, most yeast bioassays are based on an extra-chromosomal reporter construct with β-galactosidase as a substrate based reporter protein[52,53,54,55,56]. The best known ones are the YES screen developed by Routledge and Sumpter[52] and the yeast estrogen bioassay developed by McDonnell and Gaido[53]. Both assays use yeast cells that express the human ERα and contain an ERE-lacZ expression plasmid. However, in the YES assay the hERα is continuously expressed while in the assay of McDonnell and Gaido 0.05 mM of $CuSO_4$ has to be added to induce the expression of the hERα by the CUP1 metallothionein promoter. Although human cell lines are more sensitive, yeast based assays have several other advantages. These include low costs and easier handling, lack of known endogenous receptors that may compete with the activity under investigation (crosstalk), the difficulty to prepare media that are devoid of steroids[57,58,59], and last but not least, yeast cells are extremely robust and survive extracts from dirty sample matrices such as sediments[59]. That yeast cells may not be able to identify compounds that require metabolism for activation into their active state, can be an advantage rather than a disadvantage, as demonstrated by the activities of E2, E1 and E3 in the ER-CALUX which were discussed above. Also, the fact that pure antagonists like ICI 182,780 often fail to show their antagonistic effect in yeast cells, can, in the routine use to screen for estrogenic substances, be seen as an advantage as well. This because the insensitivity of yeast cells to anti-estrogens will lower the chance on false negative results.

The second major aim of the research in this thesis was to develop, validate and apply a new recombinant yeast screen to detect chemicals with an estrogenic mode of action. The development of the recombinant yeast cells that express the human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) as a reporter protein in response to estrogens (REA), is described in Chapter 3. GFP is a protein that exhibits green fluorescence that can be measured directly[60]. This yEGFP can be measured much easier than the β-galactosidase, used in the first generation of yeast assays. Furthermore, compared to other yeast assays, these new cells contain both the receptor construct as well as the reporter construct stably integrated in the genome. The robustness and ease of the yeast cells in combination with the qualities of yEGFP, ensure that the assay will be suited to be used as a high through-put system. In Chapter 4, the properties of this newly developed RIKILT yeast Estrogen bioAssay (REA) were further studied by testing a series of estrogenic compounds. Furthermore, a similar assay was developed based on the stable expression of human estrogen receptor β (hERβ). In Chapter 6 the REA was validated for the screening of estrogenic activity in calf urine. In Chapter 7 the REA was validated for the screening of estrogenic activity in animal feed. In addition, two incidents with animal feed, in which the RIKILT yeast estrogen bioassay was responsible for the detection of estrogenic substances, are described in Chapter 7.

Thus, altogether, after this introduction in Chapter 1, Chapter 2, 3 and 4 of this thesis describe the development and optimisation of three reporter gene bioassays, whereas Chapter 2, 5, 6 and 7 describe the validation and performance of these newly developed assays in both the routine screening of foods and feed for quality control and during incidents with dioxins and hormones. The summary in Chapter 8 concludes about the overall performance of the REA and DR-CALUX® bioassays, describes several other short-term incidents in which the DR-CALUX® bioassay was used successfully by RIKILT, shows additional data for the performance of the REA for the screening of estrogenic residues in calf urine, describes the future developments with both bioassays and shortly describes the development and application of a new RIKILT yeast Androgen bioAssay (RAA).

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2

Validation and use of the CALUX-bioassay for the determination of dioxins and PCBs in bovine milk

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Summary

There is a strong need for the development of relatively cheap and rapid bioassays for the determination of dioxins and related compounds in food. A newly developed CALUX (Chemical-Activated LUciferase gene eXpression) bioassay was tested for its possible use to determine low levels of dioxins in bovine milk. Data show that this mammalian cell-based test is very sensitive for 2,3,7,8-substituted dioxins and related PCBs, thereby reflecting the relative potencies of these compounds in comparison to TCDD (TEF-values). The limit of detection was about 50 fg of TCDD. Furthermore, the response obtained with a mixture of dioxins was additive, in accordance with the TEF-principle.

Milk fat was isolated by centrifugation followed by clean-up of the fat with n-pentane, removal of the fat on a 33% H_2SO_4 silica column, and determination of Ah receptor agonist activity with the CALUX-bioassay. An equivalent of 67 mg fat was tested per experimental unit, resulting in a limit of quantification around 1 pg i-TEQ/g fat. To investigate the performance of the method, butter fat was cleaned and spiked with a mixture of 17 different 2,3,7,8-substituted PCDD and PCDF congeners at 1, 3, 6, 9, 12 and 15 pg TEQ/g fat, as confirmed by GC/MS. In this concentration range, the method showed a recovery of TEQs around 67% (58-87%). The reproducibility, determined in three independent series showed a CV varying between 4% and 54%, with the exception of the sample spiked at 1 pg i-TEQ (CV 97%). The repeatability determined with the sample spiked at 6 pg i-TEQ/g showed a CV of 10%.

Testing of 22 bovine milk samples, taken at different sites in The Netherlands, in the CALUX-assay showed combined dioxin and dioxin-like PCB levels equivalent to 1.6 pg TCDD/g fat (range 0.2-4.6). GC/MS analysis of these samples revealed an average level of 1.7 pg i-TEQ/g fat, varying between 0.5-4.7 pg i-TEQ/gram fat. All five samples showing a GC/MS determined dioxin content of more than 2 pg i-TEQ/g fat, gave a response in the CALUX-assay corresponding with more than 2 pg TCDD/g fat.
These data clearly show that the CALUX-bioassay is a promising for the rapid and low cost screening of dioxins in bovine milk.

1. Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) represent a class of widespread environmental contaminants (Safe et al. 1985, 1986, 1987, 1990, Ahlborg et al., 1992). Exposure to specific PHAHs results in a wide variety of species- and tissue-specific toxic and biological effects, including hepatoxicity, birth defects, immunotoxicity, tumor promotion, changes in hormone metabolism, and enzyme induction (Safe, 1986, Poland and Knutson, 1982, Brouwer, 1991, Giesy et al., 1994, Safe, 1994). Since these effects already occur at low doses, relatively low residue levels are permitted for certain food items. Therefore, sophisticated, laborious and expensive analytical methods have been developed.

Most of the effects of these compounds are mediated by their ability to bind to the soluble cellular aromatic hydrocarbon receptor (AhR). This ligand-receptor complex is transformed and subsequently translocated to the nucleus, where it binds with high affinity to DNA at specific so-called dioxin responsive elements (DREs), stimulating transcription of adjacent genes (Denison and Yao, 1991, Denison and Whitlock, 1995). Normally this results in an increased transcription of genes coding for enzymes, such as the cytochrome P450 IA subfamily and UDP-glucuronyl transferase (De Vito and Birnbaum, 1994). The cytochrome P450 IA-dependant O-deethylation of ethoxyresorufin (EROD) is often used as an *in vivo* and *in vitro* biomarker for exposure and in particular for the determination of dioxin levels in various samples (Sawyer et al., 1984, Brouwer, 1991, Tillitt et al., 1991, Jones et al., 1993, Kennedy et al., 1993, Eggens et al., 1995). Based on the same mechanism, a new bioassay was recently developed, using the gene coding for firefly luciferase as a reporter gene (Aarts et al., 1995). A vector containing the luciferase gene under transcriptional control of DREs isolated from the 5'-flanking region of the mouse P450 IA1 gene, was stably transfected into the H4IIE rat hepatoma cell line, already containing the various factors involved in the Ah receptor pathway. It was shown that the induction of luciferase activity in these cells exposed to TCDD is dose-dependent and can be detected at lower levels than in the case of the EROD activity (Aarts et al., 1995). Furthermore, for the PCDD-, PCDF-, and PCB-congeners tested thusfar, the potency to induce CALUX activity relative to TCDD is in accordance with reported TEF values (Aarts et al., 1995, Garrison et al., 1996, Sanderson et al., 1996).

The aim of the present study was to investigate whether the CALUX-bioassay can be used for the detection of low levels of dioxins in milk. A previously developed relatively simple and fast procedure for the preparation of suitable extracts, based on the removal of fat on a 33% H_2SO_4 silica column, was further optimized (Aarts et al., 1996, Bovee et al., 1996, Murk et al., 1996). To validate the CALUX-

bioassay in combination with this clean-up procedure, a comparison was made between CALUX and GC/MS determined levels of dioxins in butter fat samples spiked at 1 to 15 pg i-TEQ per gram fat. Furthermore, 22 bovine milk samples collected in 1997 from different regions in The Netherlands were investigated by both methods.

2. Materials and Methods

Materials

The pGudLuc 1.1-transfected H4IIE cells were obtained from the Department of Toxicology, Agricultural University, Wageningen. Fetal calf serum (FCS) was purchased from Gibco BRL (Breda, The Netherlands), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from Schmidt BV (Amsterdam, The Netherlands), α-MEM, penicillin/streptomycin, bovine serum albumin from Sigma (St. Louis, MO, U.S.A.), dimethylsulfoxide (Uvasol-grade), n-pentane (p.a.), n-hexane (p.a.), diethyl ether (p.a.), cyclohexane (p.a.), anhydrous sodium sulphate (dried for 16 hours at 150°C prior to use) and silica gel 60 (70-230 mesh) from Merck (Darmstadt, Germany), ethylacetate (HPLC-grade) from Acros (Geel, Belgium) and activated carbon from Anderson (AX21). Two dioxin mixtures containing respectively 2.0 and 0.2 ng each of the 17 different 2,3,7,8-congeners of PCDD/F per ml (in toluene) were prepared from standards obtained from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.). Native butter fat came from a batch with a dioxin content of 6 pg i-TEQ/g, used at RIKILT-DLO as a control sample in the analysis of dioxins. Bovine milk samples were collected at different sites in The Netherlands, nine of them in a range from 500 to 1000 meters from municipal waste incinerators.

Preparation of spiked butter fat samples

A sample of 200 g fat was dissolved in 200 ml distilled n-hexane at 50°C, mixed with 4 g activated carbon and stirred for 1 hour. The carbon was removed by filtering the solution twice over a Whatman 41 Ashless filter (ϕ 9 cm), and twice over a Whatman 41 Ashless filter containing anhydrous sodium sulphate. The solution was evaporated to dryness under vacuum and subsequently under nitrogen. An aliquot of 36.6 g fat was dissolved in ethylacetate/cyclohexane (1:1, v/v) and mixed with 800 μl of a solution containing 2.0 ng dioxins per ml, in order to obtain a concentration of 125.8 pg i-TEQ/g fat. This highly contaminated batch was subsequently diluted with clean butter fat, aiming at six different batches with dioxin levels of respectively 1, 3, 6, 9, 12 and 15 pg i-TEQ/g fat.

GC/MS analysis

Dioxins and PCBs in milk were determined by GC/MS as described previously (Tuinstra et al., 1994). In short, fat was collected from milk samples by centrifugation for 10 minutes at 2000 g. The fat was spiked with ^{13}C-standards (internal standards) and mixed thoroughly with 25 grams of anhydrous sodium sulphate. The fat was extracted with 60 ml n-pentane and the extract was filtered over glass mineral wool with anhydrous sodium sulphate. This extraction step was repeated until the pentane remained colourless. The filter was washed with n-pentane and the combined filtrate was evaporated to dryness under vacuum and subsequently under nitrogen. The fat was removed on a gel-permeation column and the fraction containing the dioxins and PCBs was further purified on an Al_2O_3 and subsequently a graphitized carbon column. The contents of the 17 2,3,7,8-substituted dioxins, 3 planar PCBs (PCB #77, #126 and #169) and a number of mono- and di-ortho-substituted PCBs (PCB #28, #52, #101, #118, #138, #153 and #180) in the 22 field samples were determined by GC/MS.

Standards used for determining CALUX-TEFs were dissolved in DMSO, mixed with related ^{13}C-labelled standards and diluted in toluene, prior to GC/MS analysis.

Clean-up of milk samples for the CALUX-bioassay

Fat was isolated as described above, omitting the addition of ^{13}C-labelled standards. An aliquot of 0.50 g fat was dissolved in 2 ml distilled n-hexane/diethyl ether (97:3, v/v). Glass columns (ϕ 0.8 cm) containing glass mineral wool at the bottom, were packed with 6 g 33% H_2SO_4 silica and some anhydrous sodium sulphate on top. The columns were prerinsed with respectively 10 ml n-hexane and 10 ml n-hexane/diethyl ether (97:3, v/v), and subsequently the samples were quantitatively transfered to the columns, which were than eluted with n-hexane/diethyl ether (97/3, v/v). The eluate was collected starting from the moment of sample application and subsequently reduced to less than 2 ml by rotor-evaporation at 50°C. The extract was dried under a gentle flow of nitrogen and 20 μl of DMSO was added just prior to the complete evaporation of the solvent. Aliquots of 10 and 5 μl were mixed with 2 ml of medium, in the latter case followed by the addition of 5 μl DMSO.

To investigate the stability and recovery of pure standards, a 20 μl sample of the standard mixture containing 0.2 ng each of the 17 different 2,3,7,8-congeners of dioxins per ml in toluene was diluted in n-hexane/diethyl ether (97:3, v/v) and 2 ml samples were extracted with the 33% H_2SO_4 silica procedure.

CALUX-bioassay

Rat H4IIE hepatoma cells, stably transfected with an AhR-controlled luciferase reporter gene construct (pGudLuc1.1), were cultured in α-MEM culture medium supplemented with 10% (v/v) FCS, 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were grown confluent in 24-multi well plates (Costar) and exposed in triplicate to standards, mixtures of standards or milk extracts for 20-24 hrs. Each well contained 0.5 ml medium including 0.5% (v/v) DMSO, used as a vehicle. Standards were diluted in DMSO and subsequently dissolved in the medium. In the case of the mixture of dioxins, a 1 ml sample of the pure mixture containing 0.2 ng/ml each of the 17 different 2,3,7,8-dioxin congeners in toluene was evaporated to a volume of about 50 μl under a gentle flow of nitrogen. Just before the extract was completely dried, 1 ml DMSO was added and the remaining toluene was evaporated. From this stock solution (containing 0.57 ng i-TEQ per ml DMSO), dilutions in DMSO were prepared and subsequently diluted in medium at two different dilutions.

Following exposure, cells were washed three times with 0.5 ml PBS and lysed in 75 μl lysis reagent (Promega). After 15 minutes the cell lysates were transferred into tubes and frozen at -80°C. For determination of the luciferase activity the samples were thawed on ice and centrifuged for 3 minutes at 13,000 g. An aliquot of 20 μl supernatant was pipetted into a 96-well microtiter plate. The luciferase activity was determined using a Luminoskan RS luminometer (Labsystems) which automatically injected 100 μl of a luciferin assay mix (Promega) just prior to the measurement. The protein content was determined in a microtiter plate according to Bradford (1976) using protein assay dye reagent (Biorad) and bovine serum albumin (BSA) as a standard.

Statistics

The luciferase activity, expressed in relative light units (RLUs), was corrected for the protein content of the cell lysates, but in general this had no major impact on the results. Although not observed in the present studies, a decreased protein-content would have been regarded as a sign for cytotoxicity, caused by the test compound or milk extract. For determination of the TCDD equivalency factors for PHAH compounds in the CALUX-bioassay (CALUX-TEFs), a complete dose-response curve in triplicate was established for both TCDD as well as the compound of interest. Dose-response curves were fitted using a one-ligand curve fit (SlideWrite Plus Version 6.00; formula y=a0*x/(a1+x)), according to the formula:

$$\text{response} = (\text{max.response}) \times [\text{agonist}] / (EC50 + [\text{agonist}]),$$

where: (response) is the luciferase concentration, as determined by its activity; [agonist] is the concentration of the test compound; (EC50) is the concentration of agonist giving a half maximal response. The TCDD equivalency factor (CALUX-TEF) was calculated by dividing the EC50 value for TCDD by the EC50 value for the compound of interest. Concentrations of the stock solutions were checked by GC/MS and corrected if necessary.

TEQs from GC/MS data were calculated using published consensus TEF values (NATO/CCMS, 1988, Ahlborg et al., 1994,). CALUX based dioxin levels were calculated by comparison of the luciferase activity induced by a sample extract against a dose-response curve generated from TCDD standards included in each test. Again, the standard curve was fitted using the one-ligand curve fit (SlideWrite Plus 6.00). Mean and standard deviation (SD) values were calculated from individual pg TCDD eq./g fat values thus obtained from each of the three replicates, after correction for the content of the non-spiked butter fat.

Responses of cells treated with different concentrations of TCDD were tested for significancy using the Student's t-test.

3. Results

Dose-response of the most important dioxins and PCBs in milk

Exposure of pGudLuc1.1-transfected H4IIE cells to TCDD resulted in a dose-related increase in luciferase activity (Figure 1). The dose-response curve for TCDD usually started to rise around 1 pM of TCDD and reached a plateau around 100 pM, best described by an one-ligand curve-fit formula. In this particular case, the EC50, i.e. the concentration giving a half-maximum response, was calculated to be 7

pM. Figure 1 also shows the dose-response curves for a number of other selected dioxins and PCBs. The shape of the curves for these compounds was comparable to that of TCDD, but shifted to a higher dose range. For most compounds, the maximum response was comparable, with the exception of PCB 126 showing a 20% higher response, and PCBs 105 and 118 with a maximum at 60-70% of that for TCDD. The two di-ortho-substituted PCBs 170 (tested up to 50 μM), and 180 (tested up to 500 μM) failed to show a clear positive response. EC50 values determined for the different dioxin and PCB congeners are summarized in Table 1, as well as the CALUX-TEFs, calculated from these EC50 values. In all cases the concentrations of the stock solutions in DMSO were determined by GC/MS analysis, using an appropriate ^{13}C-labelled standard. For the more potent agonists, there was a good correlation between i-TEFs and CALUX-TEFs. However, for weak agonists, such as the di-ortho PCBs, CALUX-TEFs were orders of magnitude lower than the i-TEFs.

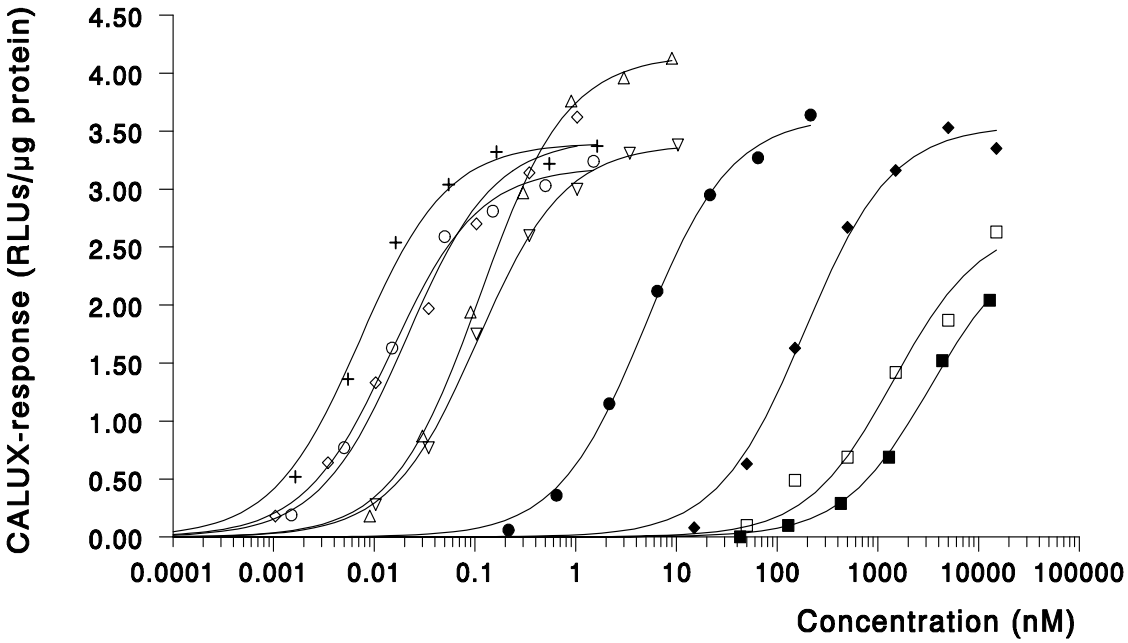

Figure 1. Dose-response curves obtained in the CALUX-assay with a number of different dioxins and PCBs: 2,3,7,8-TCDD (+), 1,2,3,7,8-PeCDD (○), 2,3,4,7,8-PeCDF (◊), 1,2,3,6,7,8-HxCDD (▽), 3,3',4,4',5-PeCB (△), 3,3',4,4',5,5'-HxCB (●), 2,3,3',4,4',5-HxCB (♦), 2,3',4,4',5-PeCB (□) and 2,3,3',4,4'-PeCB (▪). Curves were fitted using a one-ligand curve-fit, following correction of the concentrations for the GC/MS determined levels of the stock solutions (see Table 1). Data points are means of 3 independent measurements.

Table 2 shows the CALUX determined TEQ levels for six different concentrations of a mixture containing equal amounts of the 17 different 2,3,7,8-congeners of PCDDs and PCDFs. Based on i-TEF values, 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD and 2,3,4,7,8-PeCDF contribute for respectively 45, 22 and 15% of the TEQ content in this mixture, with another 15% covered by the seven hexachloro PCDD/F congeners. As shown in Table 2, the CALUX-determined concentrations are comparable to those calculated using the i-TEFs.

Limits of detection and quantification

Figure 2 shows the dose-response curves for TCDD as obtained in six independent experiments over a period of 2 months. In addition to the blank, twelve different TCDD concentrations were tested, spread over a low-dose (0-10 pM) and a high dose (0-500 pM) multiwell plate. The standard deviation as calculated over triplicates per concentration was generally less than 5%. Table 3 shows the CALUX-response obtained in these studies with TCDD concentrations in the range of 0.27 to 1.64 pM. With the

exception of the second study, a concentration of 0.27 pM TCDD gave a significantly elevated response and can be regarded as the limit of detection. Based on the sample volume of 0.5 ml per well, this concentration corresponds to an absolute amount of TCDD of 0.135 fmol or 43 fg. In the case of milk fat, an absolute amount of 67 mg fat is tested per well. Combined, this results in a theoretical limit of quantification of 0.64 pg TCDD or TEQ per gram fat.

Table 1. Toxic Equivalency Factors (CALUX-TEFs) for induction of luciferase in H4IIE-pGudLuc1.1 cells of a number of selected dioxins and PCBs.

Compound		EC50 (pM)*	i-TEF	CALUX-TEF
2,3,7,8-TCDD		7	1	1
1,2,3,7,8-PeCDD		15	0.5	0.49
2,3,4,7,8-PeCDF		21	0.5	0.34
1,2,3,6,7,8-HxCDD		106	0.1	0.068
3,3',4,4',5-PeCB	PCB 126	111	0.1	0.065
3,3',4,4',5,5'-HxCB	PCB 169	4.8×10^{3}	0.01	1.5×10^{-3}
2,3,3',4,4',5-HxCB	PCB 156	1.9×10^{5}	5.0×10^{-4}	3.8×10^{-5}
2,3',4,4',5-PeCB	PCB 118	1.4×10^{6}	1.0×10^{-4}	4.9×10^{-6}
2,3,3',4,4'-PeCB	PCB 105	3.3×10^{6}	1.0×10^{-4}	2.1×10^{-6}

*EC50 values were corrected for the GC/MS determined concentrations, being respectively 109, 69, 100, 71, 60, 43, 100, 86 and 100% of the values calculated from the preparation of the stock solutions.

Repeatability

In order to test milk fat samples in the CALUX-assay, it was necessary to extract the dioxins from the milk fat. For this purpose a clean-up procedure based on the use of a column filled with 33% H_2SO_4 silica was used. For testing the characteristics of the assay in combination with the extraction procedure, six different batches of butter fat were prepared. A batch of butter fat was cleaned with activated carbon and subsequently spiked with the mixture containing equal amounts of the 17 different 2,3,7,8-substituted dioxins at levels of 1, 3, 6, 9, 12 and 15 pg TEQ/g fat. These concentrations were confirmed by GC/MS analysis.

The repeatability of the method was investigated in two different studies. In the first study aliquots of the six different spiked butter fats and the cleaned butter fat were extracted in duplicate and tested in one CALUX-assay. Table 4 shows the dioxin content calculated for the different samples after correction for the blank fat. For most samples small variations were obtained for the duplicate samples. In a second experiment, four aliquots of the butter fat spiked at 6 pg TEQ/g fat and one aliquot of the blank fat were extracted and tested in one CALUX-assay. In this study the coefficient of variation was calculated to be 9.7%.

Table 2. CALUX-determined dioxin concentrations of medium samples spiked with different levels of a pure mixture containing equal amounts of the 17 different 2,3,7,8-substituted PCDDs and PCDFs. Two samples were tested after extraction with a 33% H_2SO_4 silica column.

	CALUX determined concentration (pM TCDD eq.)		CALUX determined concentration after extraction (pM TCDD eq.)	
Calculated concentration (pM i-TEQ)	Mean ± SD	Recovery (%)	Mean ± SD	Recovery (%)
0.39	0.74 ± 0.08	191		
1.17	1.50 ± 0.10	129		
2.33	2.57 ± 0.32	110	1.83 ± 0.17	78
3.49	3.35 ± 0.29	96		
4.66	5.89 ± 0.13	126		
5.82	6.66 ± 0.54	114	4.29 ± 0.36	74

Results are shown as the mean ± SD (n=3). Recoveries are calculated in comparison to the calculated i-TEQ concentration. When compared to the non-extracted samples, the acid silica treatment resulted in recoveries of respectively 71 and 64% for the 2.33 and 5.82 pM i-TEQ samples.

Reproducibility

The reproducibility of the method was tested in three independent tests with the spiked batches 1 to 6 and the blank batch. In each test 0.5 g of each batch of butter fat was purified and subsequently tested in the CALUX-assay, including a full TCDD standard curve. The absolute responses obtained for the undiluted extracts and a number of selected TCDD concentrations are shown in Figure 3. In general there was a dose-related increase in the CALUX-response obtained with each series of samples. After fitting the data of the TCDD standard curves, the dioxin content of the spiked butter fat batches was calculated from their luciferase activity and corrected for the non-spiked blank fat. As shown in Table 5, the CV for the three independent test series varied between 4 and 54%, with the exception of the sample spiked at 1 pg i-TEQ/g (CV 97%).

In most cases the two-fold diluted sample extracts also showed an elevated response. In comparison to the concentrations calculated from the CALUX-response of the undiluted sample, the concentrations calculated from the two-fold diluted samples were respectively 83, 88 and 120% in the three different series (sample spiked at 1 pg i-TEQ/g excluded). No clear dose-relationship was observed for the differences in the results obtained with diluted and two-fold diluted samples.

Recovery

Application of the extraction procedure on two different levels of a standard mixture, i.e. in the absence of fat, resulted in recoveries of 71 and 65% (Table 2), as compared to the non-extracted mixtures. As shown in Tables 4 and 5, the CALUX determined dioxin content in spiked milk fat gave recoveries around 60 to 67%.

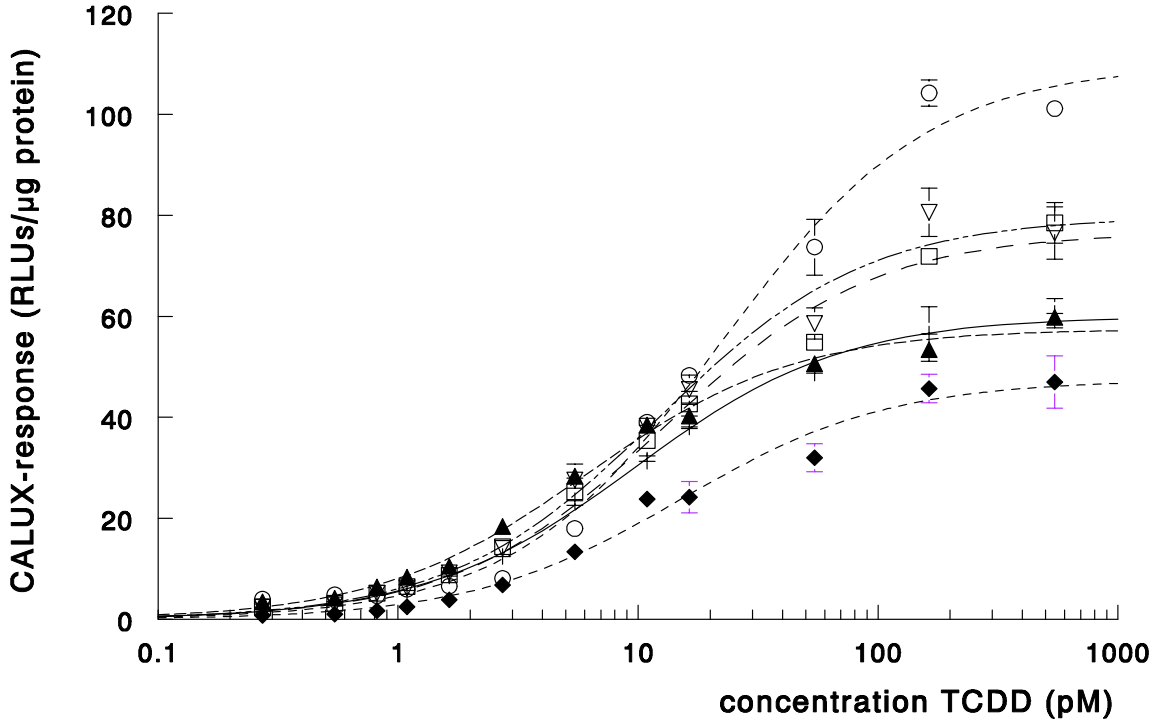

Figure 2. TCDD response curves obtained in six independent experiments. Curves were fitted using a one-ligand curve-fit.

Table 3. CALUX-responses obtained for low concentrations of TCDD in six different studies.

Concentration (pM)	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6
0	2.5 ± 0.1	3.8 ± 0.3	2.2 ± 0.1	1.7 ± 0.0	1.9 ± 0.1	0.5 ± 0.0
0.27	3.0 ± 0.1*	4.0 ± 0.5	3.5 ± 0.1*	2.4 ± 0.1*	2.5 ± 0.3*	0.8 ± 0.1*
0.55	3.5 ± 0.5*	4.9 ± 0.3*	4.2 ± 0.2*	2.9 ± 0.1*	3.2 ± 0.2*	1.0 ± 0.1*
0.82	4.7 ± 0.3*	4.7 ± 0.3*	6.4 ± 0.5*	5.2 ± 0.5*	5.2 ± 0.5*	1.7 ± 0.1*
1.09	5.7 ± 0.1*	6.0 ± 0.3*	8.4 ± 0.5*	6.2 ± 0.4*	6.5 ± 0.1*	2.5 ± 0.2*
1.64	8.6 ± 0.6*	6.6 ± 0.7*	10.5 ± 1.5*	9.2 ± 0.8*	8.8 ± 0.8*	3.9 ± 0.2*

Results are expressed as RLUs/µg protein (mean ± SD for triplicate samples). Responses indicated with * are sigificantly elevated over the response of the untreated cells ($p<0.05$, Student's t-test).

GC/MS and CALUX determined TEQ contents in 22 cows milk samples

The procedure was subsequently used to test 22 different milk samples that were collected during 1997 from different sites in The Netherlands. Contents of the 17 2,3,7,8-substituted PCDD and PCDF congeners, 3 planar PCBs and several mono- and di-ortho-substituted PCBs were determined by GC/MS analysis. Using the i-TEFs shown in Table 1, this revealed an average dioxin content of 1.7, varying between 0.5 and 4.7 pg i-TEQ/g fat. PCB 126 was the most important agonist, contributing on average for 66% (range 56-85%) to the total TEQs. The remaining fraction was covered by 2,3,4,7,8-PeCDF (16.9%), PCB 118 (7.2%), 1,2,3,7,8-PeCDF (2.7%), 2,3,7,8-TCDD (2.1%), 1,2,3,7,8-HxCDF (1.0%) and 1,2,3,6,7,8-HxCDD (0.9%).

Table 4. Repeatability of CALUX-determined levels of dioxins in spiked butter fat. In the first experiment duplicate samples of the six spiked butter fats were extracted and tested in one CALUX-assay. In the second experiment the cleaned butter fat and four samples of the fat spiked at 6 pg i-TEQ/g were extracted and tested in one CALUX-assay.

Dioxin-content (pg i-TEQ/g)	CALUX determined dioxin-content (pg TCDD eq./g)							Recovery
					Mean	SD	CV (%)	(%)
experiment 1								
1.0	0.8	0.3			0.6			60
3.0	2.5	2.2			2.4			80
6.0	3.6	3.0			3.3			55
9.0	5.6	5.1			5.4			60
12.0	6.3	6.5			6.4			53
15.0	9.2	7.5			8.4			56
experiment 2								
6.0	4.8	5.6	5.5	4.6	5.1	0.5	9.7	85

* Values were corrected for the blank (non-spiked butter fat) which were determined to contain respectively 1.4, 1.2 pg TCDD eq./g fat for the first experiment and 1.9 pg TCDD eq./g fat for the second.

The 22 samples were extracted and divided over two different series. Figure 4A shows the CALUX-response of these two series in comparison to the GC/MS determined levels. These results clearly indicate that the first series gave on average a higher response, despite the relatively low GC/MS determined levels. Using the TCDD calibration curves, the CALUX-response was converted to TCDD eq.-levels and corrected for the blank (Figure 4B). The combined figures showed a correlation between CALUX and GC/MS determined dioxin levels of 0.74. Neither the GC/MS analysis, nor the CALUX assay revealed any samples that exceeded the residue limit of 6 pg i-TEQ/g fat. Using an arbitrary cut-off point of 2 pg i-TEQ/g fat (two times the limit of quantification), all 5 samples exceeding this level according to GC/MS, also showed an increased CALUX-response. All these samples were collected around waste incinerators. Two additional samples, not collected around waste incinerators, gave a CALUX-response higher than 2 pg i-TEQ/g fat but a GC/MS determined level below 2 pg i-TEQ/g fat.

Discussion

The results from the present study demonstrate that the CALUX-assay is a very promising tool for screening milk samples on the presence of dioxins and dioxin-like PCBs. The very high sensitivity of the assay (Figures 1 and 2, Table 2) allows the clean-up of relatively low amounts of fat. Secondly, the different agonists show a relative potency which reflects the i-TEFs set for these compounds (Table 1), and mixtures of agonists show additivity, which is the basis for the TEF-principle. This is in agreement with the good correlation between the affinity of dioxin and PCB congeners for binding to the Ah-receptor and their potency to induce EROD and AHH activity in mammalian cells, as well as the good correlation between these effects and the toxic potency of these compounds (Safe et al. 1987). An exception are the mono-ortho PCBs which give a relatively weak response (Table 1), and in particular the di-ortho-substituted PCBs 170 and 180, which gave no clear response. However, at a recent WHO-meeting in Stockholm, it was decided to abolish the TEF values for these latter two compounds, which is clearly supported by the present data. In these experiments it turned out to be essential to check the actual concentrations of the standards of dioxins and PCBs, which in some cases were below 50% of the expected value (Table 1).

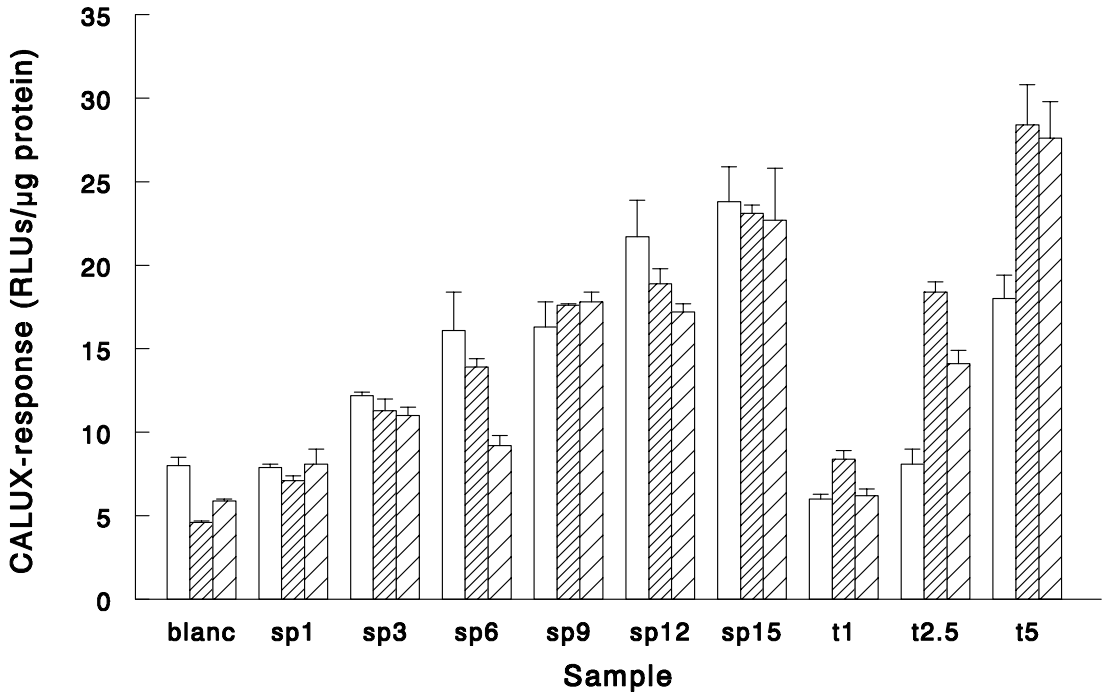

Figure 3. CALUX-response obtained with undiluted extracts from cleaned butter fat (blank) and the same fat spiked at 1, 3, 6, 9, 12 and 15 pg i-TEQ/g (sp 1 to sp 15), as obtained in three independent experiments. For comparison the results for three concentrations of TCDD, 1 (t1), 2.5 (t2.5) and 5 (t5) pM are included.

Based on the analysis of the 22 field samples in this study, the first 5 compounds in Table 1 account for 90% of the TEQ levels in bovine milk. As a result of the differences between i-TEFs and CALUX-TEFs for these compounds, the use of the CALUX-assay would theoretically result in 20% lower TEQ levels, when compared to the data from a GC/MS analysis. In the case of the standard mixture used in this study for spiking the butter fat, a reduction of 8% is expected. This might partly explain the lower recoveries obtained with the spiked milk samples (Tables 4 and 5).

The data on the reproducibility and repeatability of the CALUX-assay in combination with the acid silica clean-up procedure, further support the suitability of the assay. Even in this relatively small concentration range (1-15 pg TEQ/g fat) the test performed reasonably well, in most cases resulting in a correct ranking of samples (Tables 4 and 5). The results clearly demonstrate that the reproducibility of the silica-CALUX procedure with samples containing more than 1 pg iTEQ per g fat is relatively good, in particular regarding the fact that no internal standards could be used in the bioassay for correction of data for varying recoveries. The fact that the CV was much higher for the sample spiked at the lowest level confirms the calculated limit of quantification of 1 pg TEQ/g.

The high sensitivity of the bioassay requires the use of very pure chemicals and solvents. The presence of contaminants most likely explains the relatively high background signals observed for cleaned butter fat in two of the five experiments performed in this study (Figures 3 and 4). Inclusion of a blank sample allows the correction for these impurities. In addition, it seems appropriate to include a reference sample spiked at the maximum residue limit with a mixture of the most important dioxins and PCBs.

The tolerance for bovine milk in the Netherlands is 6 pg TEQ per g fat, Therefore, the CALUX-bioassay can be a useful pre-screening tool for selecting milk samples that may contain dioxin levels exceeding this tolerance. This is supported by the results obtained with the 22 field samples, since neither the GC/MS analysis, nor the CALUX-assay resulted in any samples exceeding this tolerance level (Figure 4). The latter data confirm that the sensitivity of the test may actually be around 2 pg i-TEQ/g fat, since all five samples exceeding this concentration gave a higher response in the CALUX-assay. The fact that only two additional samples exceeding 2 pg i-TEQ/g fat, gave a GC/MS determined content below this limit, confirms the value of the test for selecting a set of samples that may require further investigation.

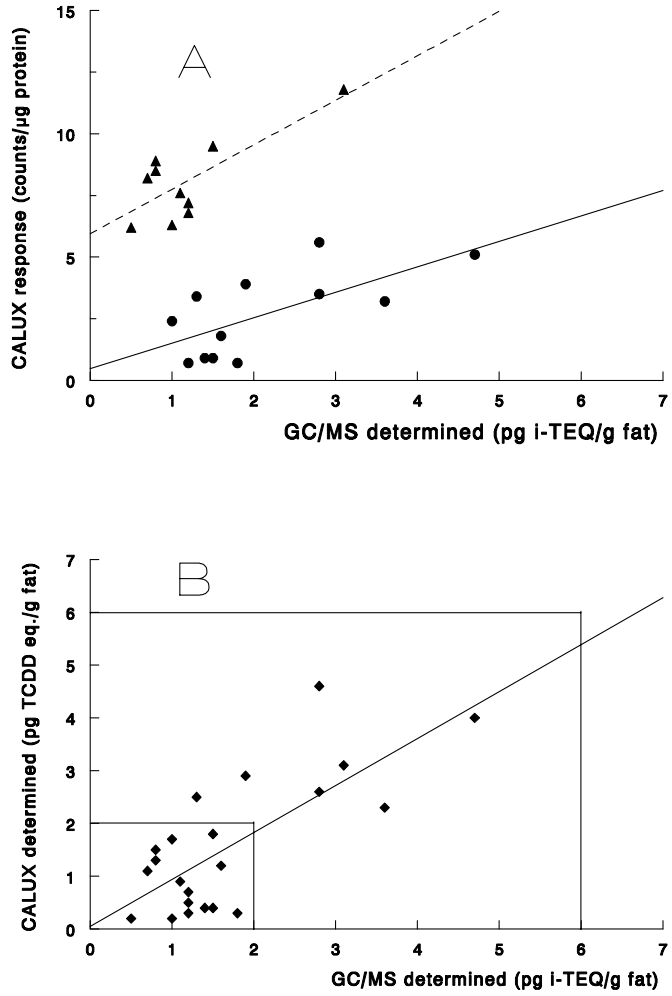

Figure 4. A comparison of (A) the CALUX-response or (B) the corresponding dioxin-content, with the GC/MS-determined TEQ content determined for 22 different milk samples collected during 1997 at sites near waste incinerators in The Netherlands. Samples were extracted and tested in two different series (A: ○---○: test 1, ●---●: test 2). Linear regression lines for the two separate series (A) or the combined series (B) are included. In the case of the GC/MS determined TEQ-content, PCBs are included.

A clear difference between a bioassay and an analytical method is the specificity of the method. The specificity of the GC/MS method for dioxins and PCBs is high due to a combination of a very sophisticated clean-up procedure and the use of high-resolution mass-spectometry. In theory, the CALUX-assay may respond to any compound capable of activating the Ah-receptor. Furthermore, any compound that interferes with one of the steps in the signal transduction pathway may limit or enhance the response of the test. Thusfar the assay does not seem to suffer from inhibition of the indicator enzyme, as observed in the case of the EROD-assay (Murk et al., 1996a, Hoogenboom and Hamers, 1995). Coexposure of cells with Ah-receptor agonists and di-ortho PCBs has been shown to result in a reduced CALUX-response (Aarts et al., 1995). However, such interactions were only observed at medium concentations around 10 µM and higher, under the present conditions corresponding to concentrations of about 25 µg PCB/g fat. As shown from the 22 field samples and other reports (Krokos et al., 1996), levels of these PCBs observed in milk are in the lower ng/g fat range, i.e. too low for interfering with the assay. Glucocorticoid hormones, and in particular dexamethasone, have been shown to increase the cytochrome P450 1A induction by TCDD in isolated hepatocytes (Donato et al., 1990,

Devaux et al. 1992) and H4IIE cells (Schrenk et al., 1991, Wiebel and Cikryt, 1990). This effect was shown to be associated with an increased expression of the Ah receptor (Wiebel and Cikryt, 1990, Abbott et al., 1994). Studies with dexamethasone and other corticosteroids revealed that this group of compounds is also capable of increasing the CALUX-response for TCDD by a factor of two (Hoogenboom et al. 1998). In the case of the thusfar most potent compound, dexamethasone, the effect was observed at a medium concentration of 1 nM, corresponding to a fat concentration of about 2.5 ng/g fat. At present it is unclear whether these compounds would actually be present in milk and in particular in the extracts prepared from milk fat, regarding the relatively destructive nature of the 33% H_2SO_4 used in the procedure. Furthermore, the interference of this group of compounds can be excluded by the inclusion of dexamethasone in the incubation medium, at a concentration that gives a maximum response (Hoogenboom et al., 1998).

Table 5. Reproducibility of CALUX-determined levels of dioxins in spiked butter fat. Samples of the six spiked butter fats were extracted three times in three independent series and tested in three independent CALUX-assays.

Dioxin-content	GC/MS determined dioxin level	CALUX determined dioxin level* (pg TCDD eq./g)					Recovery
(pg i-TEQ/g)	(pg i-TEQ/g)	Series 1	Series 2	Series 3	Mean ± SD	CV (%)	(%)
1	1.0	0.0	0.8	1.0	0.6 ± 0.6	97	60
3	3.0	2.7	2.5	2.5	2.6 ± 0.1	4	87
6	6.0	5.4	3.6	1.6	3.5 ± 1.9	54	58
9	9.0	5.5	5.6	6.5	5.9 ± 0.6	10	66
12	12.0	9.6	6.3	6.1	7.4 ± 2.0	27	62
15	15.0	11.4	9.2	10.0	10.2 ± 1.1	11	68

The contents were corrected for the levels determined for the non-spiked butter fat, being respectively 4.5, 1.4 and 2.5 pg TCDD eq./g fat for series 1, 2 and 3.

In addition to certain dioxins and PCBs, a number of polycyclic aromatic hydrocarbons (PAHs), like benzo(a)pyrene, have been shown to give a positive response in the CALUX-assay (Bovee et al., 1996, Hoogenboom et al., 1998). However, at low concentrations the effect was only observed when the cells were exposed for only 4 hrs. This is probably due to the biotransformation of these compounds by the cells, in combination with the continuous degradation of the luciferase in the cells. Another group of compounds, showing a slight positive response, were the benzimidazole drugs, used as anthelmintics in veterinary practice (Hoogenboom and Hamers, 1995). Based on these data, concentrations of e.g. 0.1 μg oxfendazole/g fat could result in a positive response in the CALUX-assay. Again, it is unknown whether these compounds would be present in the final extracts. Furthermore, the use of these drugs in dairy cows is not allowed.

These data clearly demonstrate that the selection of specific test conditions in combination with the clean-up procedure results in a highly specific test. Furthermore, the possible presence of benzimidazole drugs or glucocorticoids would result in a higher CALUX-response, which to a certain extent is acceptable for a screening assay. False-negative results, due to general cytotoxicity, can be detected by a decrease in the routinely measured protein content of the cell-lysates. More subtle inhibitory effects might be detected by testing compounds or food extracts also in the presence of a positive control, like TCDD.

It can be concluded that the CALUX-assay is a very valuable tool, allowing the screening of relatively large sets of samples for the presence of unacceptably high levels of dioxins and dioxin-like PCBs. This will result in a considerable reduction of the costs involved in the analysis of food for the presence of these compounds.

Acknowledgements

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Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein

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Abstract

The aim of this study was to develop an estrogen transcription activation assay that is sensitive, fast and easy to use in the routine screening of estrogen activity in complex matrices such as agricultural products. Recombinant yeast cells were constructed that express the human estrogen receptor α (ERα) and β-Galactosidase (βGal), Luciferase (Luc) or yeast Enhanced Green Fluorescence Protein (yEGFP) as a reporter protein. Compared to other yeast assays, these new cells contain both the receptor construct as well as the reporter construct stably integrated in the genome with only one copy of the reporter construct. Dose-response curves for 17β-estradiol (E2) obtained with the βGal assay were similar to those reported and the calculated EC50 of 0.2 nM was even slightly better. However, 5 days of incubation were required before the chlorophenol red product could be measured. The Luc assay was as sensitive as the βGal assay and gave an EC50 of 0.2 nM, but the signals were rather low and, although the assay can be performed within one day, the procedure is laborious and caused variability. The yEGFP revealed an EC50 of 0.4 nM, but compared to the βGal and the Luc assay, the response was much better. This yEGFP assay can be performed completely in 96 well plates within 4 hours and does not need cell wall disruption nor does it need the addition of a substrate. This makes the test sensitive, rapid and convenient with high reproducibility and small variation. These qualities make that this yEGFP assay is suited to be used as a high throughput system.

1. Introduction

Estrogens influence the growth, differentiation and function of many target organs, such as the mammary gland, uterus, vagina, ovary, testis, epididymis and prostate. Estrogens also play an important role in bone maintenance, the central nervous system and in the cardiovascular system (Schomberg et al., 1999, Couse and Korach, 1999, Wang et al., 2003). Most effects of estrogens are thereby mediated by estrogen receptors (ER). After binding of a ligand to the ER, dissociation of heat shock protein 90 (Hsp 90) enables occupied ERs to dimerise. The resulting homodimer complex exhibits high affinity for specific DNA sequences, referred to as estrogen responsive elements (EREs), located in the 5' regulatory region of estrogen inducible genes. The ligand occupied ER-dimer functions as a transcription factor that modulates the activity of these responsive genes (McDonnell et al., 1995).

During the past decades, a large number of structurally diverse chemicals have been released into the environment. Many of these chemicals and waste products have steroid-like activity and accumulate in the air, water and food chain. By acting as estrogen mimics (xenoestogens), they may disrupt normal endocrine function, possibly leading to reproductive failure in humans and wildlife and tumours in estrogen sensitive tissues (Sharpe and Skakkebaek, 1993, Pike et al., 1993, Guillette et al., 1994, Jobling et al., 1998, Tyler et al., 1998). Due to the great variety of chemicals with estrogen-like activity, classical instrumental analysis is not the most suitable tool to assess the estrogenic potency of complex mixtures like food samples. Therefore a number of in vivo and in vitro assays have been developed. In vivo assays, such as the mouse uterotrophic assay, are highly valuable to assess the overall biological effect of a compound. However, due to high costs, labour intensiveness, relatively poor sensitivity and modest responsiveness, in vivo assays are generally unsuitable for large-scale screening. Furthermore, in vivo approaches are not capable of identifying endocrine mechanisms for the observed effects. In vitro assays, such as competitive ligand binding assays, cell proliferation and estrogen receptor transcription assays, are more suited. However, competitive ligand binding assays can not distinguish between receptor agonists and antagonists. Cell proliferation assays, such as the E-screen (Soto et al., 1995), use ER-positive, estrogen-responsive MCF-7 (E-screen) or T47-D human breast cancer cells. But MCF-7 cells also express androgen, progesterone, glucocorticoid and retinoid receptors. This may compromise the suitability of the assay if substances are also able to bind to other receptors. It has e.g. been shown that androgens, progestins and glucocorticoids can antagonise E2-induced cell proliferation. Furthermore, proliferative responses occur only after a number of days (Korach et al., 1995, Ramamoorthy et al., 1997, Zacharewski, 1997).

More recently, genetically modified yeast cells and human cell lines are being used for estrogen activity measurement by transcription activation of reporter genes. These systems can also be used to identify ER antagonists by giving them in combination with a near maximally effective dose

of E2. Although human cell lines are more sensitive than yeast and may be able to identify estrogenic compounds that require human metabolism for activation into their estrogenic state (Legler et al., 1999, Hoogenboom et al., 2001), yeast-based assays have several advantages. These include robustness, low costs, lack of known endogenous receptors and the use of media that are devoid of steroids. Until now, yeast estrogen bioassays are based on an extra-chromosomal reporter construct with β-Galactosidase as a substrate based reporter protein (Routledge and Sumpter, 1997, Gaido et al., 1997, Rehmann et al., 1999, Morito et al., 2001, Le Guevel and Pakdel, 2001). Alternative reporters are Luciferase, which has been used as a highly sensitive reporter in animal cells (Aarts et al., 1995, Bovee et al., 1998, Legler et al., 1999) and Green Fluorescent Protein (GFP). GFP is a protein that exhibits green fluorescence that can be measured directly (Cormack et al., 1997). This paper reports the development of yeast estrogen bioassays by creating stably transfected strains with βGal, Luc or yEGFP as measurable reporter proteins. The correct functioning of the developed bioassays was confirmed by exposure studies with 17β-estradiol in 50 ml polypropylene tubes and test protocols were then optimised for exposures in 96 wells format to select an assay that is most suited to be used as a high throughput system.

2. Materials and Methods

2.1. Chemicals

Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Difco (Detroit, MI, USA), cell culture medium D-MEM/F-12, foetal bovine serum (FBS) and Trizol reagent from Gibco BRL (Life Technologies Ltd, Paisley, Scotland) and 17β-estradiol, L-histidine, L-leucine and uracil from Sigma (St. Louis, MO, USA). Ammonium sulphate, chloroform, isoamyl alcohol, isopropanol, ethanol absolute and dimethyl sulfoxide were obtained from Merck (Darmstadt, Germany), zymolyase-100T from ICN (Costa Mesa, CA, USA) and Deoxyribonuclease I and Ribonuclease inhibitor from Promega (Madison, WI, USA). All restriction endonucleases and corresponding buffers were obtained from New England Biolabs (NEB, England, UK). The T47D human breast cancer cell line was provided by Dr B. van der Burg (NIOB, Hubrecht Laboratorium, Utrecht, the Netherlands).

2.2. Yeast strain

The yeast Saccharomyces cerevisiae (CEN.PK 102-5B, K20, URA3 $^{-}$, HIS3 $^{-}$, LEU $^{-}$) was a gift from H. Silljé (University of Utrecht, the Netherlands).

2.3. Plasmids

For the expression of the human estrogen receptor α, the p403-GPD yeast expression vector described by Mumberg et al. 1995 was used. For construction of the reporter plasmid, the p406-CYC1 yeast expression vector described by Mumberg et al. 1995 was used. Both plasmids were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The pyEGFP3 plasmid was a gift of A.J. Brown. The pGL3-Basic Vector (LUC+) and the pSV-β-Galactosidase Control Vector were purchased from Promega (Madison, WI, USA).

2.4. Isolation of mRNA

Human breast cancer cells T47D were grown in 75 cm^2 cell culture flasks in DMEM-F12 medium containing 7.5% FBS. Cells were grown confluent in the flasks, washed with PBS-Ca-Mg, released with 0.25% trypsin/0.05% EDTA and harvested in 3 ml PBS-Ca-Mg. To collect the cells, an aliquot of 1ml was centrifuged at 4,000 g for 1 min. The pellet was frozen on liquid nitrogen and stored at -80°C. To isolate the mRNA, 1 ml Trizol was added to an unfrozen cell pellet, mixed for 30 s and incubated at room temperature for 5 min. The solution was centrifuged at 12,000 g for 15 min at 4°C and the supernatant was transferred to a clean tube. Addition of 0.5 ml chloroform/isoamyl alcohol (24:1 v/v) was followed by 15 s mixing and a 3 min incubation at room temperature. This solution was centrifuged at 12,000 g for 15 min at 4°C and the aqueous phase was transferred to a clean tube. The RNA was precipitated by addition of 0.5 ml isopropanol, 15 s head over head mixing, 10 min incubation at room temperature and centrifugation at 12,000 g for 15 min at 4°C. The RNA pellet was washed with 1 ml ice-cold 75% ethanol and after centrifugation at 12,000 g for 10 min at 4°C, dried for 10 min in a vacuumexcicator. The dry RNA pellet was dissolved in 50 μl DEPC treated ultra pure water for 10 min at 55°C. Traces of DNA were removed by the addition of 3 μl DNase I (Deoxyribonuclease I, 134 U/μl), 7 μl RNasin (Ribonuclease Inhibitor), 7 μl 10x concentrated DNase buffer, 3 μl DEPC

treated ultra pure water and an 1 h incubation at 37°C. Subsequently, the DNase was inactivated for 10 min at 65°C.

2.5. Synthesis of cDNA

Synthesis of cDNA was carried out on the T47 D mRNA using the Advantage RT-for-PCR Kit (Clontech) with the MMLV Reverse Transcriptase and the random hexamer primers. The protocol of the supplier was used and the synthesised cDNA of the T47D human breast cancer cells was stored at -80°C.

2.6. Isolation of full length human estrogen receptor α cDNA

Full length human ERα cDNA was obtained from the T47 D cDNA with a PCR using the Expand High Fidelity PCR System (Boehringer Mannheim). Conditions were: 34.2 μl ultra pure water, 5 μl 25 mM $MgCl_2$, 5 μl Expand HF 10x concentrated buffer (without $MgCl_2$), 0.8 μl 25 mM dNTP mix, 1 μl of the enzyme mix, 2 μl T47D cDNA and 2 μl of a primer mix containing 10 μM of each primer were pipetted into a thin-walled PCR tube. PCR was performed in an Eppendorf Mastercycler gradient using the following cycle profile: 1) denature template 3 min at 95°C; 2) denature template 30 s at 94°C; 3) anneal primers 1 min at 58°C; 4) elongation 2 min at 72°C; 5) go to step 2 and repeat 31 times; 6) elongation 7 min at 72°C and step 7) for ever 10°C. The sequence of the 5'-primer was: 5'-GCGGATCCATGACCATGACCCTCCACAC-3' containing a restriction site for BamH I just before the ATG start codon. The sequence of the 3'-primer was: 5'-GCGAATTCGGGAGCTCTCAGACTGTGGC-3' containing a restriction site for EcoR I just after the TGA stop codon. This PCR generated a full-length ds cDNA of 1812 bp of the human ERα gene with a 5'-BamH I and a 3'-EcoR I restriction site.

2.7. Construction of the p403-GPD-ERα receptor expression vector

The 1812 bp full length ERα PCR product (see sections 2.4. to 2.6.) was isolated from a 1% low-melt agarose gel using a QIAquick Gel Extraction Kit according the manufacturers (Qiagen) protocol using a microcentrifuge. This ERα cDNA was ligated into a pGEM-T Easy Vector (Promega) and this vector was used to transform Epicurian Coli XL2-Blue Ultracompetent Cells (Stratagene). Both ligation and transformation were performed according to the manufacturers protocols. Following transformation, 100 and 900 μl samples of the culture were plated on LB agar plates with 100 μg/ml of ampicillin, 80 μg/ml XGal and 0.5 mM IPTG. Plates were incubated at 37°C overnight and single white colonies were streaked out on fresh plates. Plasmid isolation of inoculated 3 ml LB cultures containing 100 μg/ml ampicillin was performed according to the manufacturers QIAprep 8 Miniprep Kit Protocol (Qiagen). Plasmid digestion control with EcoR I revealed several clones with the correct DNA fragments of 3015 and 1812 bp. One of these clones was sequenced in both directions using the SEQ 4x4 apparatus and the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit, all used according to the manufacturers instructions (Amersham Pharmacia). All 1788 base pairs, from the ATG start to the TGA stop, corresponded to the human estrogen receptor α sequence published by Greene et al. (1986). This pGEM-T Easy-ERα clone was used to construct the p403-GPD-ERα expression vector. Cleavage with BamH I and EcoR I gave the ERα DNA fragment that was ligated into the corresponding site of the p403-GPD vector. This p403-GPD-ERα vector was used to transform Epicurian Coli XL-2 Blue Cells. Plasmid digestion controls and PCR controls of single white colonies were performed and revealed several good clones (data not shown).

2.8. Construction of the p406-ERE_2-CYC1 reporter vectors

Two sets (S1 and S2) of complementary oligonucleotides (a and b), each with two consensus ERE-sequences (in bold), were synthesised. A solution with both complementary DNA sequences, 2.5 μM of each, was heated at 95°C and cooled down to room temperature in 2 hours. Set 1 gave ds DNA with two consensus EREs and 5'-Sac I and 3'-Sph I sticky ends. Set 2 gave ds DNA with the same two consensus EREs and a 5'-Sac I sticky end and a 3'-Msc I blunt end, compared to set 1, set 2 restores part of the CYC1 promoter. Oligonucleotides:

S1a) 5'-AAAGTCAGGTCACAGTGACCTGATCAAATCTAGAAGATCCAAAGTCAGGTCACAGTGACCTGATCAAACATG-3'
S1b) 5'-TTTGATCAGGTCACTGTGACCTGACTTTGGATCTTCTAGATTTGATCAGGTCACTGTGACCTGACTTTAGCT-3'
S2a) 5'-AAAGTCAGGTCACAGTGACCTGATCAAATCTAGAAGATCCAAAGTCAGGTCACAGTGACCTGATCAAACTCGAGCA
GATCCGCCAGGCGTGTATATATAGCGTGGATGG-3'
S2b) 5'-CCATCCACGCTATATATACACGCCTGGCGGATCTGCTCGAGTTTGATCAGGTCACTGTGACCTGACTTTGGATCTT
CTAGATTTGATCAGGTCACTGTGACCTGACTTTAGCT-3'

Both sets were cloned into the corresponding sites of the p406-CYC1 vector. Ligation and transformation were performed in a similar way as described earlier for the construction of the p403-GPD-ERα expression vector (see section 2.7.). Digestion and PCR controls revealed several good clones for both the p406-ERE2s1-CYC1 and the p406-ERE2s2CYC1 reporter construct. In the same way, yEGFP, Luciferase and β-Galactosidase, obtained from a Hind III and Sal I double digestion of respectively pyEGFP, pGL3-Basic Vector (Luciferase) and pSV-β-Galactosidase Control Vector, were cloned in the corresponding Hind III/Sal I sites of both p406-ERE2-CYC1 reporter constructs. In this way two different reporter constructs were constructed, different in the way the ERE2 was placed in the CYC1 promoter, and both with three different reporter genes: p406-ERE2s1-CYC1-yEGFP, p406-ERE2s1-CYC1-Luc, p406-ERE2s1-CYC1-βGal, p406-ERE2s2-CYC1-yEGFP, p406-ERE2s2-CYC1-Luc and p406-ERE2s2-CYC1-βGal. Plasmid digestion controls and PCR controls revealed several good clones for each constructed plasmid (data not shown).

2.9. Transformation of yeast cells

Transformation of yeast K20 (Ura $^-$, His $^-$ and Leu $^-$) was performed by the Lithium-Acetate protocol (Short protocols in molecular biology, Chapter 13.7). First, this yeast was transformed with the six different reporter vectors (see section 2.8.), integrated at the chromosomal location of the Uracil gene via homologous recombination. Therefore, prior to transformation, the reporter vectors were linearised by cutting with Stu I, which has a unique restriction site in the URA3 marker gene. Transformants were grown on MM/LH plates. PCR and Southern blot hybridisation were used to select clones in which the integration has occurred at the desired URA3 site with only a single copy of the reporter vector (see sections 2.10. and 2.12.). In a similar way, these six different reporter strains were then each transformed with the p403-GPD-ERα expression vector, which was linearised by cleavage with Bbs I, which has a unique restriction site in the HIS3 marker gene (Histidine). Transformants were grown on MM/L plates and Western blots and PCR controls were used to select clones that expressed the human estrogen receptor α (see sections 2.11. and 2.12.).

2.10. Southern blot

Yeast chromosomal DNA of reporter transformants was isolated by the method of Hoffman and Winston, (1987) (see Short protocols in molecular biology, Chapter 13.11). The DNA was digested with a) Hind III, b) Sal I and c) a double digestion of Hind III and Sal I. Digestion reactions were run on a 1% agarose gel in TBE buffer and blotted onto a nylon Hybond-N+ membrane (Amersham Pharmacia Biotech) with an alkaline buffer (see Short protocols in molecular biology, Chapter 2.9). DNA is cross-linked to the membrane by 2 h heating at 80°C under vacuum. Probes were made by Hind III and Sal I double digestions of pyEGFP, pGL3-Basic Vector (Luciferase) and pSV-β-Galactosidase Control Vector and the digestion reactions were run on a 1% agarose gel in TBE. The corresponding bands of respectively yEGFP (736 bp), Luciferase (1957 bp) and β-Galactosidase (3749 bp) were excised from the gel with a scalpel. The DNA was extracted and purified with a QIAquick Gel Extraction Kit. From this DNA, probes were made with the Prime-It II Random Primer Labeling Kit (Stratagene) using d*CTP primer buffer and α32PdCTP (3000 Ci/mmol) according to the manufacturers instructions. The probes were purified with the QIAquick Nucleotide Removal Kit (Qiagen) according to the manufacturers protocol. Hybridisation was performed overnight in 30 ml 5x Denhardts, 6x SSC, 0.5% SDS in a hybridisation oven at 65°C. The blots were washed and an autoradiograph was made overnight at -80°C using a Kodak Biomax MS-1 film (Sigma) or a Fuji Medical X-Ray film.

2.11. Western blot

Western blot analysis was performed according to an adapted protocol described by Laemmli et al., 1970. Briefly: proteins from yeast cytosensors that contained the p403-GPD-ERα expression vector were isolated by centrifugation of 1 ml of the yeast culture and resuspending the cell pellet in 0.5 ml sample buffer (20 mM Tris/HCl pH 6.8, 0.8% (w/v) SDS, 3.5% (v/v) glycerol, 0.002% (w/v) bromophenolblue, 2% (v/v) β-mercaptoethanol). Samples were shaken for 45 min and 0.25 g glass beads were added (425-600 microns, acid-washed, Sigma). Samples were vortexed three times for 1 min, heated at 95°C for 5 min, centrifuged at 13,000 g for 5 min and 20 μl of the supernatant was loaded on a 10% SDS polyacrylamide gel. The gel was run at 120 V until the loading dye ran off the gel (approximately 90 min). Proteins were transferred to a Millipore Immobilon-P PVDF membrane (0.45 μm) at 120 V for 60 min at approximately 5°C (using BioRad mini-PROTEAN II gel-electrophoresis and wet-electroblotting apparatus). The membrane was air dried, soaked in 100% methanol to drive the water out and dried on Whatmann 3MM filter paper. The blot was incubated

overnight with primary antibody in blocking buffer: 0.125 ml mouse anti-ERα (mouse monoclonal IgG_{2a} 0.2 μg/μl, Santa Cruz) in 30 ml blocking buffer (PBS with 1% (w/v) BSA and 0.05% (v/v) Tween-20). The blot was washed twice with PBS for 1 min and incubated 90 min with secondary antibody in 30 ml blocking buffer: 7 μl anti-mouse Alkaline Phosphatase conjugate (1 mg/ml IgG, Promega). The blot was washed twice with PBS and once in AP-buffer. Colour development was performed in 30 ml AP-buffer (100 mM Tris/HCl pH9.5, 100 mM NaCl, 5 mM $MgCl_2$) with 200 μl NBT (50 mg/ml, Promega) and 100 μl BCIP (50 mg/ml, Promega). A protein sample of a cell pellet of human T47D breast cancer cells was prepared in the same way and was also loaded on the 10% SDS polyacrylamide gel. This sample is referred to as a positive reference sample, because these cells are known to express the estrogen receptor α.

2.12. PCR controls

PCR controls were performed on reporter and reporter/receptor transformants. Yeast chromosomal DNA was isolated by the method of Hoffman and Winston and PCR was performed with Taq DNA polymerase (Perkin Elmer). Briefly: 36.6 μl ultra pure water, 5 μl 25 mM $MgCl_2$ (PE), 5 μl 10x buffer without $MgCl_2$ (PE), 0.4 μl 25 mM dNTP mix, 1.0 μl Taq DNA Polymerase (PE), 1.0 μl yeast chromosomal DNA and 1.0 μl of a primer mix containing 10 μM of each primer were pipetted into a thin-walled PCR tube. PCR was performed in an Eppendorf Mastercycler gradient using the same cycle profile as described earlier for the PCR for the human estrogen receptor α (see section 2.6.). PCR I was performed with a 5'-primer on the backbone of the reporter plasmid and a 3'-primer on the ERE_2 sequence. However, this PCR was performed with an annealing temperature of 52 instead of 58°C. PCR II was performed with a 5'-primer on the CYC1 promoter and a 3'-primer on the CYC1 terminator. However, the cycle profile made use of an elongation step at 72°C for 5 min instead of 2 min (step 4). PCR III was performed with a 5'-primer on the human ERα and a 3'-primer also on the human ERα. PCR IV was performed with a 5'-primer on the human ERβ and a 3'-primer also on the human ERβ. PCR V was performed with a 5'primer on the GPD promoter and a 3'-primer on the CYC1 terminator. Primers:

PCR I: 5'-primer: 5'-AGCGAGTCAGTGAGCGAGGAAG-3'
and 3'-primer: 5'-CTGTGACCTGACTTTGGATC-3'
PCR II: 5'-primer: 5'-TCTATAGACACACAAACACAA-3'
and 3'-primer: 5'-GGGAGGGCGTGAATGTAAG-3'
PCR III: 5'-primer: 5'-CGAAGTGGGAATGATGAAAGGTG-3'
and 3'-primer: 5'-TGTGGGAGAGGATGAGGAGGAGC-3'
PCR IV: 5'-primer: 5'-ATGGATTGCTGCTGGGAGGAG-3'
and 3'-primer: 5'-AAGTGGGAATGGTGAAGTGTGGC-3'
PCR V: 5'-primer: 5'-CAGTTCCCTGAAATTATTCCCCTAC-3'
and 3'-primer: 5'-GGGAGGGCGTGAATGTAAG-3'

2.13. Yeast culturing conditions

Before running an assay, an agar plate containing the selective medium was inoculated with yeast from a frozen -80°C stock (20% glycerol v/v). The plate was incubated at 30°C for 24 - 48 h and then stored at 4°C. The day before running the assay, a single colony of yeast from the agar plate was used to inoculate 10 ml of the selective medium. This culture was grown overnight at 30°C with vigorous orbital shaking at 225 rpm in minimal medium (MM) containing yeast nitrogen base without amino acids or ammonium sulphate (1.7 g/l), dextrose (20 g/l) and ammonium sulphate (5 g/l). For yeast cells that were only transformed with the reporter constructs, this MM was supplemented with L-leucine (L)(60 mg/l) and L-histidine (H)(2 mg/l). For yeast cells that contained both the reporter construct and the p403-GPD-ERα expression vector, the MM was supplemented with L-leucine only. At the late log phase, the culture was diluted (1:10) into the same medium.

2.14. yEGFP assay

For yeast cells containing a yEGFP reporter construct, exposures in 96 well cell clusters (Costar) were performed with 200 μl of the diluted culture (1:10) (see section 2.13.) per well and the addition of 2 μl of a 17β-estradiol stock solution in ethanol (1% ethanol). To test the influence and the % solvent, doses of E2 in ethanol and DMSO were added (1 μl, 2 μl, 5 μl and 10 μl resulting in respectively 0.5%, 1%, 2.5% and 5 % solvent). To test lower percentages of ethanol, E2 stocks in ethanol were diluted 10 times in H2O (10% ethanol) and 4 μl and 2 μl were added to the wells, resulting in respectively 0.2 and 0.1% Ethanol. Ethanol and DMSO only controls were included in each experiment and each sample was assayed in triplicate. Exposure was performed for 4 or 24 h.

Fluorescence at these time intervals was measured directly in the CytoFluor Multi-Well Plate Reader (Series 4000, PerSeptive Biosystems) using excitation at 485 nm and measuring emission at 530 nm. The fluorescence signal was corrected with the signals obtained with the supplemented MM containing ethanol or DMSO solvent only.

2.15. Luciferase assay

For yeast cells containing a Luciferase reporter construct, exposures in the 96 well plates (1% ethanol) were performed as described for the yEGFP assay (4 and 24 h, see section 2.14.). After exposure in the 96 well plates, cells were harvested by centrifugation for 15 min at 4,000 rpm. The supernatant was removed and cell pellets were resuspended in 50 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) containing 0.1 mg/ml zymolyase. This lyticase digestion of the cell wall was performed for 1 h at room temperature (20-22°C). Protoplasts were lysed by hypoosmotic shock by the addition of 100 μl 0.1% Triton X-100 for 15 min at room temperature. Then 20 μl of the lysate was pipetted into a 96 well microtitre plate and luciferase activity was determined using a Luminoskan RS luminometer (Labsystems), which automatically injected 100 μl of a luciferin assay mix (Promega) just prior to the measurement. The luciferase activity is expressed in relative light units (RLU).

2.16. β-Galactosidase assay

For yeast cells with a βGal reporter construct, the chlorophenol red-β-D-galactopyranoside (CPRG, Roche) substrate was added to the diluted culture (1:10). The final CPRG concentration in the yeast culture was 16.5 μM. Exposure in 96 well plates was performed as described for the yEGFP assay (see section 2.14.). However, the % solvent was different as 1 μl of the E2 stock instead of 2 μl was used, resulting in 0.5% ethanol as solvent and exposure was performed for 5 days at 30°C with orbital shaking at 225 rpm. The change in concentration of chlorophenol red, the red product that results from β-Galactosidase cleavage of CPRG, was measured at OD562 nm using the Argus 400 microplate reader (Packard). This signal was corrected for cell density differences, measured as the OD at 630 nm, by dividing the OD562 by the OD630 nm.

3. Results and Discussion

Recombinant yeast cells were constructed that express the human estrogen receptor α (ERα) and β-Galactosidase (βGal), Luciferase (Luc) or yeast Enhanced Green Fluorescence Protein (yEGFP) as reporter proteins in response to exposure to estrogens. Both the receptor construct as well as the reporter construct were stably integrated into the yeast genome by the use of yeast integrating plasmids. With these plasmids, cut in their marker gene, transformation of yeast only occurs by integration into the yeast genome via homologous recombination (normally at the site of the deficient marker gene). Construction of these strains was started by the stable introduction of six different reporter vectors, integrated at the chromosomal location of the Uracil gene via homologous recombination (see section 2.9.). For the construction of these six reporter vectors the p406-CYC1 plasmid (Mumberg et al., 1995), containing the URA3 marker gene, was used. With each reporter gene, two sets of reporter vectors were made. With set 1 (s1) two consensus EREs were placed in the Sac I/Sph I site of the cytochrome-c oxidase promoter (CYC1 promoter). With set 2 (s2), the two consensus EREs were placed in the Sac I/Msc I site of the CYC1 promoter. Compared to set 1, set 2 restores the -254 to -147 Xho I - Sph I part of the CYC1 promoter (see figure 1). PCR and Southern blots (see section 2.12. and 2.10. respectively) were used to select clones with only one copy of the reporter construct.

These six different reporter yeast strains were subsequently stably transfected with the human ERα construct. High expression levels of the ERα were obtained by placing the cDNA of the human ERα gene behind the strong constitutive yeast GPD promoter in the p403-GPD plasmid. This plasmid contains the HIS3 marker gene and transfected strains (see section 2.9.) were checked with PCR and Western blots to confirm the expression of the human ERα (see section 2.12. and 2.11. respectively). The correct functioning of the cytosensors was subsequently confirmed by exposure studies with 17β-estradiol in 50 ml polypropylene tubes, using 5 ml of the diluted yeast culture and 5 μl of an E2 stock solution in ethanol (0.1%). Test protocols were then optimised for exposures in 96 wells format.

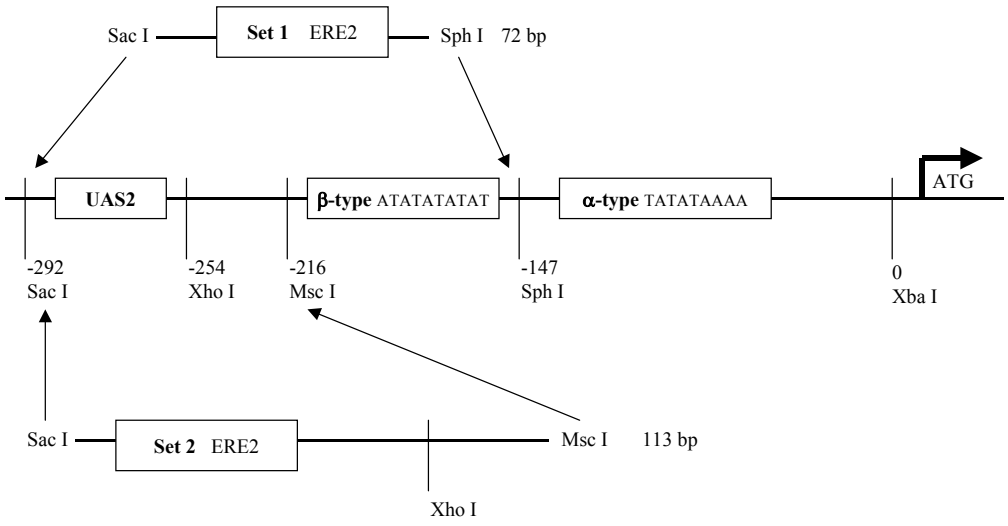

Figure 1. Schematic representation of the truncated CYC1 promoter and the construction of the s1 and s2 reporter constructs.

3.1. Southern blot on reporter constructs

Figure 2 shows the Southern blot of 7 yeast clones that were transformed with the p406-ERE$_2$s1-CYC1-yEGFP reporter construct (see section 2.10.). Clones #1, #2, #4, #5, #6, and #7 (respectively lanes 2, 3, 5, 6. 7 and 8) contain one copy of the p406-ERE$_2$s1-CYC1-yEGFP reporter construct, contrary to the yeast host (lanes 9, 10, 18 and 19). Clone #3 (lane 4) does not contain a copy of this reporter construct and was also the only one to be negative in a PCR control that was performed on isolated chromosomal DNA of these yeast clones using primers on the yEGFP gene (data not shown). Clone #1 contains one copy of the reporter construct and the double digestion of this clone (lane 20) shows the specific Hind III/Sal I fragment of 736 bp (the lowest band in the marker lane 1 is still visible on the gel and corresponds to 500 bp). This clone #1 was used for transformation with the p403-GPD-ERα expression vector. Similar studies were carried out to select yeast strains correctly expressing the other s1 reporter constructs and the s2 reporter gene constructs (data not shown).

3.2. Western blot on the ERα protein

Figure 3 shows the ERα Western blot (see section 2.11.) of yeast cells that contain a single copy of the p406-ERE$_2$s1-CYC1-yEGFP reporter construct (see section 3.1.) and that were transformed with the p403-GPD-ERα receptor expression construct (see section 2.9.). The size of the protein band in lanes 1-6 and lane 8 is about 68 kDa and corresponds to the size of the human estrogen receptor α. This Western blot clearly demonstrates that the human ERα is only expressed in the yeast cells that were transformed with the p403-GPD-ERα expression vector and not in the yeast host itself. The blot thereby proves that the hERα is faithfully expressed under the GPD promoter. The fact that the human ERα cDNA was completely sequenced in both directions and was fully complement to the sequence published by Greene et al. (1986) (see section 2.7.), proves that the expressed ERα protein, in terms of the amino acid sequence, is an exact copy of the human ERα protein.

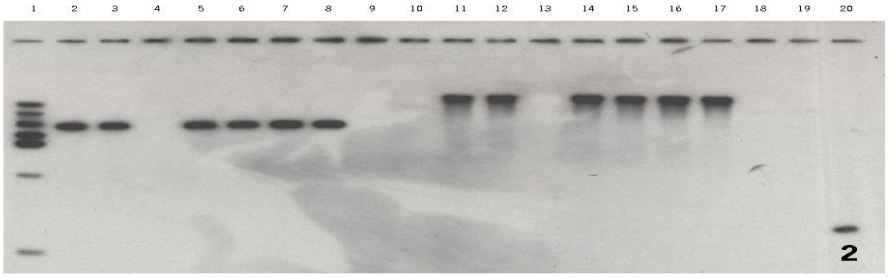

Figure 2. Southern blot of the yeast cells transformed with the p406-ERE$_2$s1-CYC1-yEGFP reporter construct. Southern blots were performed as described in Materials and Methods (section 2.10.). Lane 1 contains the marker. Lane 2-8 contain the Hind III digestion of 7 yeast clones transformed with the p406-ERE$_2$s1-CYC1-yEGFP reporter construct. Lane 9 and 10 contain the Hind III digestion of the yeast host. Lane 11-17 contain the Sal I digestion of the 7 transformed yeast clones. Lane 18 and 19 contain the Sal I digestion of the yeast host. Lane 20 contains the Hind III and Sal I double digestion of the transformed yeast clone #1. A yEGFP α32PdCTP probe of 736 bp was used for hybridisation.

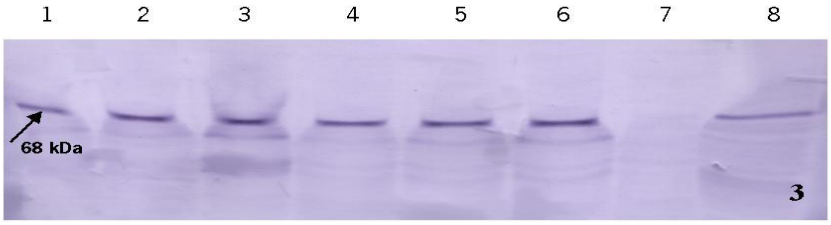

Figure 3. Western blots of yeast cells expressing the human oestrogen receptor α. Western blot was performed as described in Materials and Methods (section 2.11.) Lane 1-6 contain protein samples of the 6 yeast clones that contain one copy of the p406-ERE$_2$s1-CYC1-yEGFP reporter construct (see figure 2 clones#1, #2, #4, #5, #6 and #7). These clones were transformed with the p403-GPD-ERα receptor expression construct. Lane 7 contains the protein sample of the yeast host and is referred to as a negative control. Lane 8 contains a protein sample of human T47D breast cancer cells and is referred to as a positive control. A mouse anti-ERα monoclonal was used as primary antibody and anti-mouse Alkaline Phosphatase conjugate as secondary antibody. Colour development was performed with NBT/BCIP.

3.3. PCR controls on reporter and receptor constructs in yeast

A number of different PCR-controls were carried out to check the integration of the vectors into the yeast DNA. Figure 4 shows a number of PCR controls (see section 2.12.) that were performed on DNA samples isolated from yeast transformants that contain only the set 2 reporter gene construct for yEGFP (#1) or in combination with the p403-GPD-ERα receptor expression construct (#2). The combination with the p405-GPD-ERβ receptor expression construct (#3) is also shown. PCR controls of yeast transformants containing the set 1 reporter constructs and the set 2 reporter gene constructs for Luc and βGal are not shown.

PCR I was performed with primers on the backbone of the p406 plasmid and on the ERE_2 sequence. It gave the specific 360 bp band with DNA of yeast transformants that contain a reporter construct made with set2. PCR II was performed with primers on the CYC1 promoter and the CYC1 terminator. It gave the specific 873 bp band with DNA of transformants containing the yEGFP reporter construct. This PCR also gave a 435 bp band with the DNA of all yeast cells, this band corresponds to the CYC gene of the yeast host itself and is therefore also a specific band. PCR III was performed with primers on the human ERα gene and it gave the specific 802 bp band in all yeast cells transformed with the p403-GPD-ERα receptor expression construct. PCR IV was performed with primers on the human ERβ gene and it gave the specific 798 bp band with all yeast cells transformed with the p405-GPD-ERβ receptor expression construct. PCR V was performed with primers on the GPD promoter and the CYC1 terminator. It gave the specific 2146 bp band with yeast transformants that contain the p403-GPD-ERα receptor expression construct and the specific 1966 bp band with yeast transformants that contain the p405-GPD-ERβ receptor expression construct.

These PCR controls clearly demonstrate that all specific PCR bands can be seen. Together with the Southern blots (section 3.1.) and Western blot (section 3.2.), the PCR controls (section 3.3.) prove that the yeast cytosensors contain a single copy of the reporter construct that is stably integrated in the yeast genome. They also prove that the stably integrated human ERα construct results in the expression of the human estrogen receptor α protein in these yeast cytosensors.

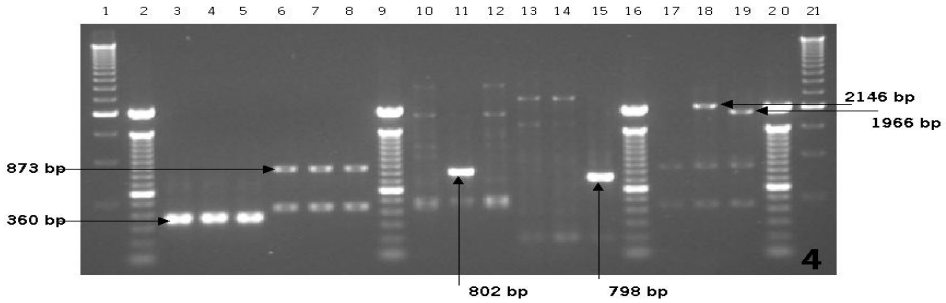

Figure 4. PCR controls performed on yeast transformants that contain the set 2 reporter gene construct for yEGFP alone (#1) or in combination with the p403-GPD-ERα receptor expression construct (#2) or the p405-GPD-ERβ receptor expression construct (#3). PCR controls were performed as described in Materials and Methods (section 2.12.). Lane 1 contains a 500 bp ladder and lane 2 contains a 100 bp ladder. Lane 3-5 are #1, #2, and #3 with PCR I. Lane 6-8 are #1, #2, and #3 with PCR II. Lane 9 contains a 100 bp ladder. Lane 10-12 are #1, #2, and #3 with PCR III. Lane 13-15 are #1, #2, and #3 with PCR IV. Lane 16 contains a 100 bp ladder. Lane 17-19 are #1, #2, and #3 with PCR V. Lane 20 contains a 100 bp ladder and lane 21 contains a 500 bp ladder.

3.4. Dose-response curves obtained with exposures in 96 well plates

Figure 5A shows results obtained from the exposure experiments in 96 well plates (see section 2.14.) with yeast cells containing either the p406-ERE_2s1-CYC1-yEGFP (set1) or p406-ERE_2s2-CYC1-yEGFP (set2) reporter construct alone, or in combination with the p403-GPD-ERα receptor expression construct. These data clearly demonstrate that yeast cells that contain only a p406-ERE_2-CYC1-yEGFP reporter s1 or s2 construct do not show a response when exposed to E2 (s1-rep and s2-rep). Exposure to E2 of yeast cells that also express the human estrogen receptor α results in a dose-related increase in fluorescence. Table 1 shows the calculated EC50 values, i.e. the concentration giving a half-maximum response, and induction factors, i.e. the fold increase (maximum of the response relative to the background), as obtained by a mathematical non-linear regression curve-fit formula. Figure 5A clearly shows that the s1-cytosensor gives very poor dose-response curves, in contrast to the s2-cytosensor which produces well-shaped curves. Although the signals after 24 h are higher than after 4 h, there are no great differences in EC50 values between 24 h and 4 h exposures. The higher signals obtained after 24 h are due to the higher number of yeast cells present in the sample (yeast growth), measured as yeast density at OD630 nm. Signals corrected for yeast density (s2-cor), by dividing the fluorescence signal by the OD630 nm, resulted in curves for 4h and

24 h that are almost the same. As the OD 630 after 24 h is about 1.0, the corrected curve (s2-cor / 24h) almost equals the not corrected curve (s2 / 24h). There are very small differences in yeast densities due to exposure to different amounts of E2. Only very high concentrations of E2 (10 nM and higher) gave small decreases in yeast densities and only after the 24 h exposure. Therefore the corrected curve (s2-cor / 24h) demonstrates a little higher induction factor (7) than the not corrected (s2 / 24h) curve (induction factor 6). Because of the small effects of yeast density, fluorescence signals obtained after exposure to E2 do not need correction for yeast density and signals are only corrected with the signals obtained from the blank medium (the sterile supplemented MM containing ethanol solvent only). This yEGFP assay is quick (4 h), sensitive (EC50 of 0.4 nM), can completely be performed in 96 well plates and does not need cell wall disruption nor does it need the addition of a substrate. These qualities make this yEGFP s2-cytosensor a promising tool for a high throughput system.

Data obtained from the exposure experiments in 96 well plates (see section 2.15.) with yeast cells containing either the p406-ERE$_2$s1-CYC1-LUC (set1) or the p406-ERE$_2$s2-CYC1-LUC (set2) reporter construct alone, or in combination with the p403-GPD-ERα receptor expression construct are shown in figure 5B. Again, yeast cells that contain only a p406-ERE$_2$-CYC1-LUC reporter s1 or s2 construct did not show a response when exposed to E2 (s1-rep and s2-rep). Exposure to E2 of yeast cells that also express the estrogen receptor α resulted in a dose-related increase in luciferase activity. Table 1 shows the calculated EC50 values and the induction factors. It is evident that, compared to the yEGFP s2-cytosensor, the dose-response curves obtained with the Luc cytosensors are relatively poor, especially after the 4 h exposure time. When comparing the s1-cytosensor with the s2-cytosensor it becomes clear that the s2-cytosensor demonstrates much lower background signals and the maximum response is also much lower (both 2 orders of magnitude, see figure 5B). However, the induction factor obtained with the s2-cytosensor is higher than that obtained with the s1-cytosensor, but the sensitivity of the s1-cytosensor is better, resulting in a lower EC50 value (see table 1). Just as for the yEGFP cytosensors, curves corrected for yeast density after 24 h of exposure did not differ from curves that were not corrected. However, curves corrected for yeast density after 4 h of exposure were still very poor (data not shown). The signals are therefore only corrected for the blank medium.

When exposures of the Luc cytosensors were performed in 50 ml polypropylene tubes and the lyticase digestion or a procedure using glass beads were used to make lysates, it became clear that using the procedure with the glass beads resulted in higher signals and better dose-response curves (data not shown). As a result, it can be concluded that the poor dose-response curves obtained with the 96 well plate method are mainly due to the poor lyticase digestion. Unfortunately, it appeared to be impossible to make a lysate with glass beads in the 96 well plate. Furthermore, it is difficult to reproducibly collect the yeast cells by centrifugation in a 96 well plate, even at a maximum speed of 4,000 rpm (1800g). Although the Luc assay with the 96 well plate method can be performed within one day, the procedure is laborious and shows very poor dose-response curves with great variability due to the above described problems with collection and disruption of the yeast cells. These properties make this Luc assay less suitable as a high throughput system.

Data obtained from exposure experiments in 96 well plates with yeast cells containing either the p406-ERE$_2$s1-CYC1-βGal (set1) or the p406-ERE$_2$s2-CYC1-βGal (set2) reporter construct alone, or in combination with the p403-GPD-ERα receptor expression construct (see section 2.16.) are shown in figure 5C. Again, the yeast cells that contain only a reporter construct do not respond when exposed to E2, but in this case also the cytosensor with the s1 reporter construct does not respond to E2. There is no explanation why this cytosensor is not working. Southern blot and PCR controls revealed the integration of both the reporter as well as the receptor construct and as a result this cytosensor was capable to grow in the selective MM/L medium. Exposure to E2 of the yeast cytosensor with the s2 reporter construct resulted in a dose-response curve. Table 1 shows the EC50 values and the induction factors.

So, the βGal assay is relatively simple and does not need cell wall disruption. However, despite the ease and the good sensitivity (EC50 of 0.2 nM) of this βGal s2-cytosensor, it takes 5 days to obtain a clear signal (with shorter exposures no dose-response could be detected). Furthermore, the variation in yeast density is greater than in the yEGFP and Luc assay, probably due to the relative long exposure of 5 days, and therefor the signal (OD562 nm) is corrected for the OD630 nm (see section 2.16.). This assay is therefor less suited as a high throughput system as such systems should normally be very quick. One of the latest redesigned protocols of a yeast estrogen βGal screen that

does not make use of a cell wall digestion or disruption, is the one described by De Boever et al. (2001), but this protocol still requires 2 days. Furthermore, this redesigned assay needs the addition of cycloheximide. After 24 h exposures, cycloheximide and the CPRG substrate were added. The cycloheximide was added to stop the protein synthesis as they found that the CPRG substrate itself displayed estrogenic activity.

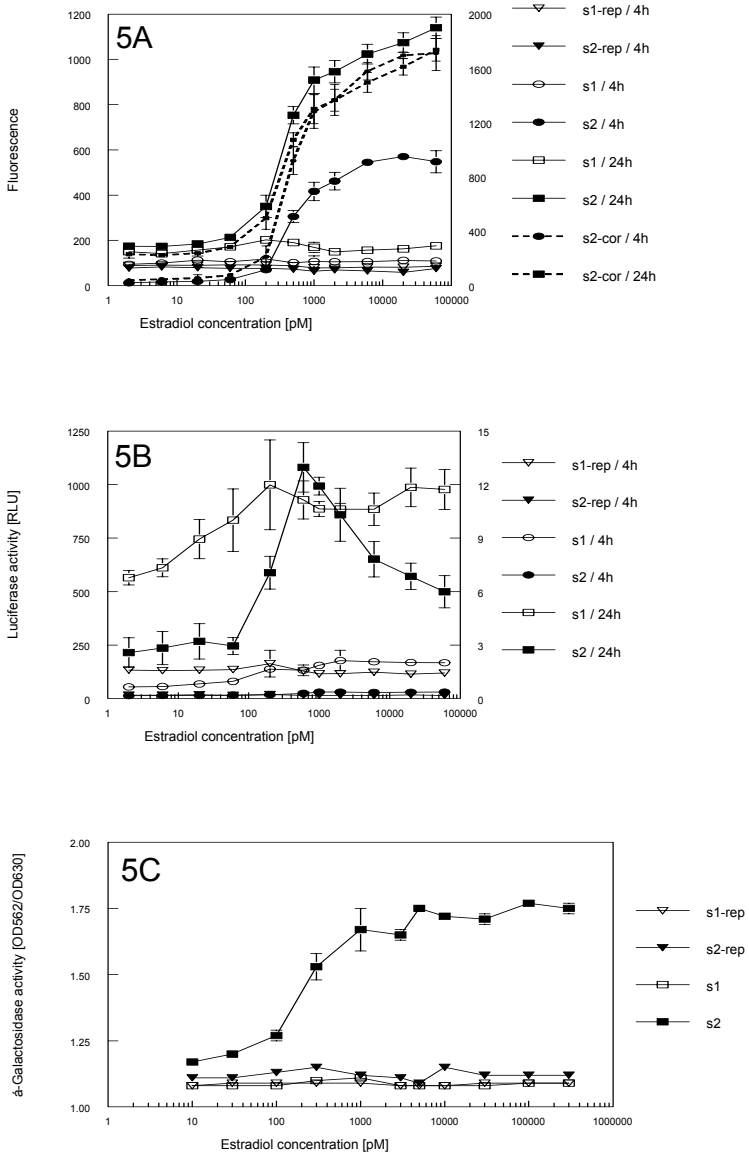

Figure 5. Exposure of yeast yEGFP (5A), LUC (5B) and βGal (5C) cytosensors in plates. Exposures in plates were performed with 200 μl of a diluted yeast culture and the addition of 1 (5C) or 2 μl (5A and 5B) of a 17β-estradiol stock solution in ethanol (respectively 0.5% for 5C and 1% for 5A and 5B). Fluorescence (4h and 24h), luciferase activity (4h and 24h) and β-Galactosidase activity (5 days) were determined as described in Materials and Methods (see sections 2.14, 2.15 and 2.16). Note that in figure 5A the for yeast density corrected signals (dotted lines) are on the right Y-axis and that in figure 5B the signals obtained with the s1-cytosensor are on the left Y-axis and those obtained with the s2-cytosensor are on the right Y-axis. Values are the mean ± sd (n=3). Open triangles are yeast cells that only contain a s1 reporter construct and open circles and open squares are the s1-cytosensors. Closed triangles are yeast cells that only contain a s2 reporter construct and closed circles and closed squares are the s2-cytosensors.

Table 1. EC50 concentrations and induction factors obtained with the yeast yEGFP, Luc and βGal cytosensors with exposures in 96 well plates

Reporter construct	Exposure time	EC50 [nM]	Induction factor
YEGFP-S1	4 h	*p.c.	
YEGFP-S1	24 h	0.06	1.3
YEGFP-S2	4 h	0.4	40
YEGFP-S2	24 h	0.4	6
YEGFP-S2-cor[1]	4 h	0.4	40
YEGFP-S2-cor[1]	24 h	0.4	7
Luc-S1	4 h	*p.c.	
Luc-S1	24 h	0.03	2
Luc-S2	4 h	*p.c.	
Luc-S2	24 h	0.2	4
βGal-S1	5 d	*n.c.	
βGal-S2	5 d	0.2	1.5

[1]fluorescence signals corrected for yeast density measured as OD630 nm
*p.c. = poor dose-response curve
*n.c. = no dose-response curve

3.5. Influence of the percentage solvent on the performance of the yEGFP assay

In the previous section it was demonstrated that the yEGFP s2-cytosensor has the best potential for use in 96 well plates as a high throughput system. In practise it would be convenient if sample extracts in ethanol or DMSO could be added in large volumes, i.e. that yeast is able to tolerate high percentages of solvent. Dose-response curves for E2 obtained with this yeast yEGFP s2-cytosensor with different percentages of ethanol or DMSO solvent and an exposure time of 4 h are presented in figures 6A and 6B respectively. Table 2 shows the calculated EC50 values and the induction factors. The best curves were obtained with 0.5% EtOH or DMSO solvent. Higher percentages of solvent gave lower signals, lower induction factors and higher EC50 values. Lower percentages of EtOH solvent (0.1 and 0.2%) gave little higher induction factors, but it also gave higher EC50 values. The differences between ethanol (figure 6A) and DMSO (figure 6B) are obvious. Compared to DMSO, ethanol as solvent resulted in lower EC50 values. Ethanol (0.5%) gave an EC50 of 0.5 nM and an induction factor of 8, while DMSO (0.5%) gave an EC50 of 0.7 nM and an induction factor of 20. Again, the signals after 24 h of exposure were higher (curves not shown), but the induction factors were lower and there were no differences in the EC50 values (see table 2). Only, the clear reduction in the sensitivity and the induction factor obtained with 2.5% DMSO with an exposure of 4 h (figure 6B) disappeared after 24 h (data not shown).

These data clearly demonstrate that it is difficult to use higher fractions of ethanol or DMSO extracts, as with both solvents 2.5% of solvent or more resulted in a reduction of the sensitivity and the induction factor of the assay. Although it is clear that ethanol instead of DMSO as a solvent resulted in lower EC50 values, it may be better to use DMSO as a solvent, because with this solvent it is much easier to work with small volumes, as this solvent does not evaporate as quick as ethanol.

3.6. Discussion

There are several yeast estrogen bioassays, most of them containing the β-Galactosidase coding sequence as a reporter gene. These yeast estrogen screens have been optimised, resulting in protocols based on the disruption of the cell wall and that are completely performed in 96 well plates in only one day. The reported EC50 values for E2 of these bioassays are between 0.1 and 3.5 nM (Routledge and Sumpter, 1997, Gaido et al., 1997; Rehmann et al., 1999, Morito et al., 2001, Le Guevel and Pakdel, 2001). In addition to these yeast estrogen screens, a number of genetically modified human cell lines are used to assess the estrogenic potency of different substances. The ER-CALUX developed by Legner et al. (1999) makes use of human T47D breast cancer cells and shows an EC50 for E2 of only 6 pM. Despite the fact that in general human cell lines are more sensitive, yeast based assays have several advantages. This includes robustness, low costs, lack of known

endogenous receptors and the use of media that are devoid of steroids. These qualities make a yeast estrogen bioassay a promising tool for the high throughput screening of relatively dirty samples, requiring little or no sample clean-up, or of complex matrices, in which there are more endocrine active substances than estrogens only. This paper describes the development of a new set of yeast estrogen screens that use yeast Enhanced Green Fluorescence Protein (yEGFP), Luciferase (Luc) or β-Galactosidase (βGal) as reporter proteins. Compared to other yeast assays, these new yeast cells contain both the receptor construct as well as the reporter construct stably integrated in the genome.

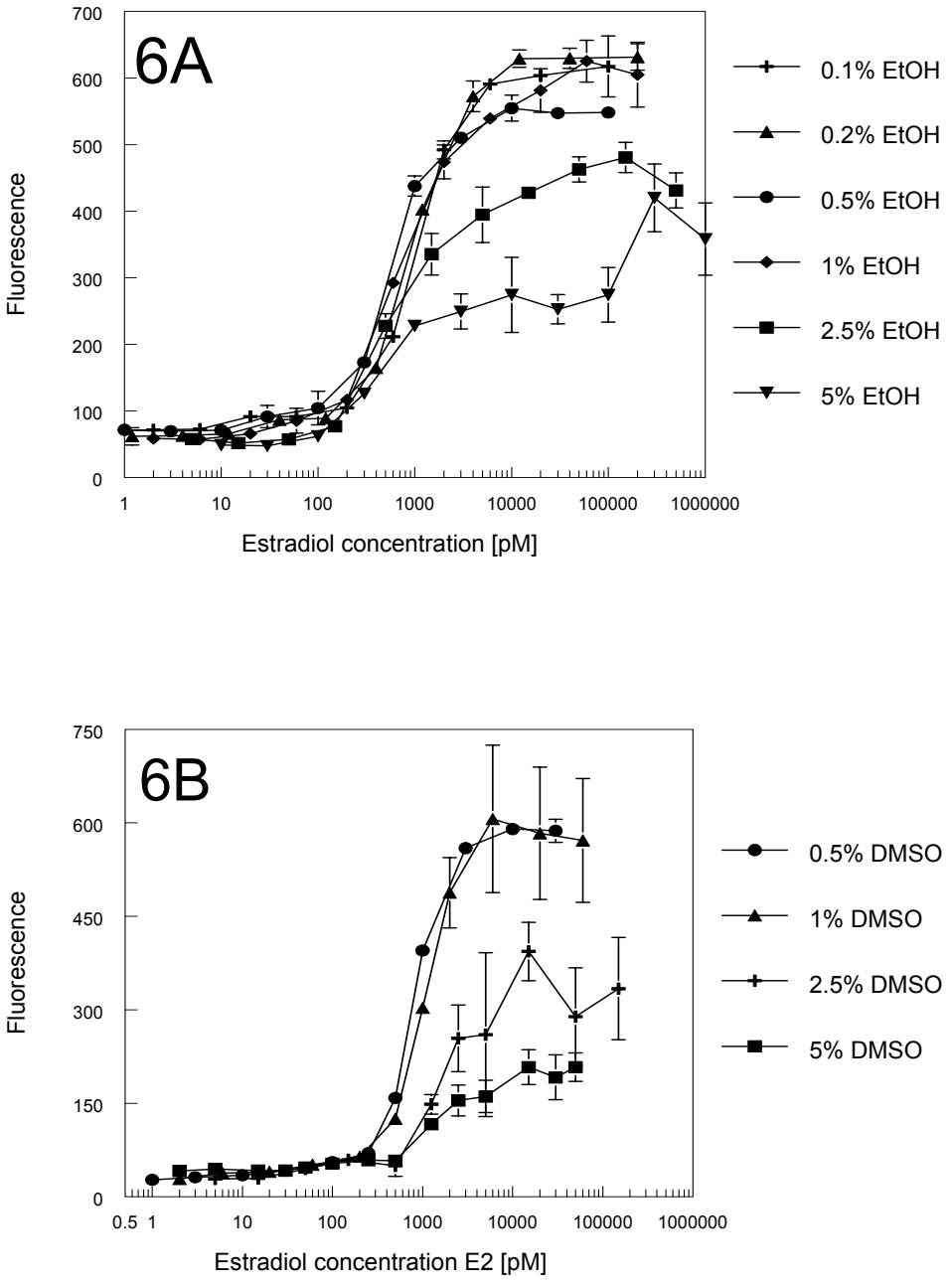

Figure 6. Exposure of 4 hours of the yeast yEGFP s2 cytosensor in plates using different % of ethanol (6A) or DMSO (6B). Exposures were performed with 200 μl of a diluted yeast culture of the yEGFP s2-cytosensor for 4h in 96 well plates and the addition of different amounts of a 17β-estradiol stock solution, resulting in different percentages of solvent. Fluorescence after 4 h exposure was determined as described in Materials and Methods (section 2.14). Values are the mean ± sd (n=3).

Table 2. EC50 concentrations and induction factors obtained with the yEGFP s2-cytosensor with exposures in 96 well plates using different % of EtOH or DMSO as a solvent

Reporter construct	Percentage solvent	Exposure time [h]	EC50 [nM]	Induction factor
S2	0.1% EtOH	4	0.8	10
S2	0.2% EtOH	4	0.8	10
S2	0.5% EtOH	4	0.5	8
S2	1.0% EtOH	4	0.7	9
S2	2.5% EtOH	4	0.6	7
S2	5.0% EtOH	4	0.8	7
S2	0.5% EtOH	24	0.5	6
S2	0.5% DMSO	4	0.7	20
S2	1.0% DMSO	4	0.9	20
S2	2.5% DMSO	4	1.6	12
S2	5.0% DMSO	4	1.3	6
S2	0.5% DMSO	24	0.7	5

The data presented in this paper clearly demonstrate that all but one of the new constructed yeast cytosensors showed a dose-related response when exposed to 17β-estradiol. In general the s2-cytosensors showed better dose-response curves than the s1-cytosensors. In the 96 well plate format the Luc s2-cytosensor showed very poor dose-response curves due to the problems with the collection and disruption of the cells. This assay appears therefor not suitable to be used as a high throughput system. The yEGFP and βGal s2-cytosensors on the other hand showed very good and sensitive dose-response curves. Although the βGal assay is very sensitive (EC50 of 0.2 nM), this assay is not very suitable as a high throughput system, since it takes 5 days to obtain a clear response and it is necessary to correct the signals for yeast density (OD630 nm). The yEGFP assay on the other hand is very sensitive (EC50 of 0.4 nM) and quick (4 h) and is at present the best high throughput assay. This new developed yeast estrogen bioassay, based on the expression of yEGFP as a reporter protein, can completely be performed in 96 well plates and the sensitivity of this yEGFP assay is comparable with that reported for βGal-based assays. This yEGFP assay can be performed within only 4 h and does not need cell wall disruption nor the addition of a substrate. The reproducibility of the assay is very good and the standard deviation is very low. Because yEGFP is measured directly in intact cells, it is possible to measure on-line. With this yEGFP assay, urine samples of calves spiked with 1 ng E2 per ml could easily be distinguished from the blank urine. These urine samples did not need any clean-up and were directly exposed to the yeast by adding 30 μl of the urine to 200 μl of the yeast suspension in a 96 well plate. Probably due to its cell wall, yeast can tolerate the salts in the urine and perform well (data not shown).

The results obtained with the yEGFP, Luc and βGal assay show that the s1 reporter construct is in general more sensitive than the s2 reporter construct, resulting in lower EC50 values for the s1-cytosensors. More important however, the curves obtained with the s2-cytosensors are much better shaped, have lower background signals and have higher induction factors. The difference between the results obtained with both reporter constructs can be explained by the way that these constructs were made. The p406-CYC1 vector contains a truncated version of the CYC1 promoter, which is no longer inducible due to deletion of the upstream activator site 1, UAS1, and deletion of most of the UAS2 sequence (Guarente and Mason, 1983; Guarente et al., 1984 and Mumberg et al., 1995). Two consensus EREs are placed in front of this truncated CYC1 promoter. The centre to centre spacing between these two consensus EREs in the s1 and s2 reporter constructs is 40 bp (see section 2.8.) because Ponglikitmongkol et al. (1990) demonstrated that paired perfect EREs stimulate transcription synergistically and in a stereo-alignment manner. Stereo-alignment is achieved if the EREs are separated by 4 or 5 integral turns and this means that the centre to centre spacing has to be 40 or 50 bp.

With the construction of the s1 reporter construct, two consensus ERE sequences were inserted into the Sac I/Sph I site of this truncated CYC1 promoter (see section 2.8. and figure 1). Compared to the yeast estrogen bioassays described by other groups, there are two main differences. First, our yeast cytosensors contain the reporter constructs stably integrated into the yeast genome instead of multicopy intact 2μ vectors. Second, the ERE2 sequence is cloned into the Sac I/Sph I site of the CYC1 promoter, and not into the Xho I site. Contrary to cloning into the Xho I site, cloning in the Sac I/Sph I site removes the last part of the UAS2 sequence and the β-type TATA element at site -178 (ATATATATAT, Li and Sherman, 1991). The α-type TATA element at site -123 (TATATAAAA) and the TATA-like sequences at nucleotides -93, -78, and -56 were not altered. Removal of the β-type TATA element and the last part of the UAS2 sequence, and the use of stably integrated reporter constructs offer no clear explanation for the relative high background signals, low induction factors and the relatively low EC50 values that were obtained with the s1-cytosensors.

According to Melcher (Melcher et al., 2000), the p406-CYC1 vector contains fortuitous activator binding sites (FABS) within the plasmid backbone. By cloning the ERE2 sequence into the Sac I/Sph I site instead of the Xho I site, as other groups did, these FABS are sited 73 bp closer to the TATA region of the promoter and the ATG start codon of the reporter gene (see figure 1). According to Melcher et al. (2000), the relative closeness of the FABS to the reporter gene can cause high background signals. This led to the decision to make the s2 reporter construct. With the s2 reporter constructs two consensus ERE sequences were inserted into the Sac I/Msc I site of the truncated CYC1 promoter by using the set 2 oligonucleotides (see section 2.8. and figure 1). In order to restore the Xho I - Msc I part of the truncated CYC1 promoter that is removed by cleavage with Sac I and Msc I, this set 2 not only contains two consensus ERE sequences, but also contains this Xho I - Msc I part of the CYC1 promoter. So, with this set 2, the truncated CYC1 promoter is restored in such a way, that it is almost the same as if the ERE2 sequence is cloned into the Xho I site of the CYC1 promoter. The only difference with other groups, that cloned into the Xho I site, is the small part of the UAS2 sequence. This UAS2 part (32 bp), between the Sac I and Xho I site, is still left out in our s2 reporter construct (see figure 1). Compared to the s1 reporter construct, which places the fortuitous activator binding sites 73 bp more closer to the reporter gene, the s2 reporter construct places these FABS 37 bp further away from the reporter gene. As a result the FABS in the s2 reporter construct are placed 110 bp further away from the reporter gene than in the s1 reporter construct. As a result the relative high background signals were reduced, but it also resulted in better-shaped curves with higher induction factors.

With the yEGFP s2-cytosensor it was also determined that 200 μl yeast suspension in the 96 well plate method gave better results than 100 μl. Furthermore, the minimal medium gave better results than a complete synthetic medium, because this complete medium gave a high background in the fluorescence measurement. In addition, it was found that the density of the yeast culture that is used for the exposure had almost no effect on the EC50-value. However, especially the induction factor after 4 h of exposure became lower with higher densities of the diluted yeast start culture, because yeast cells (even yeast cells without yEGFP) rise the fluorescence background signal.

4. Conclusions

The yEGFP protein is a very suitable marker in yeast. The new developed yeast estrogen bioassay, with yEGFP as a directly measurable reporter protein, can completely be performed in 96 well plates in only 4 hours. The EC50 of this new yeast estrogen assay is comparable with reported EC50 values for yeast estrogen bioassays that contain β-Galactosidase as a reporter. However, compared to other yeast bioassays and cell lines, this new yeast yEGFP assay is much easier and faster to perform. It does not require cell wall disruption or the addition of a substrate. As a result, this yEGFP assay is not only sensitive, resulting in an EC50 of 0.4 nM, but is also very rapid, convenient and reproducible. These qualities make this yeast estrogen yEGFP bioassay suited to be used as a high throughput system for the screening of estrogenic activity in relatively dirty samples, needing little or no sample clean-up, or complex matrices in which there are more endocrine active substances than estrogens only.

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4

Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: a comparison of different compounds with both receptor types

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Abstract

Previously we described the construction of a rapid yeast bioassay stably expressing human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens. In the present study the properties of this assay were further studied by testing a series of estrogenic compounds. Furthermore, a similar assay was developed based on the stable expression of human estrogen receptor β (hERβ). When exposed to 17β-estradiol, the maximum transcriptional activity of the ERβ cytosensor was only about 40% of the activity observed with ERα, but the concentration where half-maximal activation is reached (EC50), was about 5 times lower. The relative estrogenic potencies (REP), defined as the ratio between the EC50 of 17β-estradiol and the EC50 of the compound, of the synthetic hormones dienestrol, hexestrol and especially mestranol were higher with ERα, while DES was slightly more potent with ERβ. The gestagens progesterone and medroxyprogesterone-acetate showed no response, whereas the androgen testosterone showed a very weak response. The anabolic agent, 19-nortestosterone showed a clear dose-related response with estrogen receptor α but not β. The phytoestrogens coumestrol, genistein, genistin, daidzein, daidzin and naringenin were relatively more potent with ERβ. Ranking of the estrogenic potency with ERα was: 17β-estradiol >> 8-prenylnaringenin > coumestrol > zearalenone >> genistein >> genistin > naringenin. The ranking with the ERβ was: 17β-estradiol >> coumestrol > genistein > zearalenone > 8-prenylnaringen >> daidzein > naringenin > genistin >> daidzin. The hop estrogen 8-prenylnaringenin is relatively more potent with ERα. These data show that the newly developed bioassays are valuable tools for the rapid and high-throughput screening for estrogenic activity.

1. Introduction

There is serious concern that chemicals in our food, water and environment may affect human health by disrupting normal endocrine function. This relates both to certain chemicals with previously unknown hormonal properties, and compounds used e.g. for their growth-promoting properties in animals. On the other hand, the estrogenic properties of the soy isoflavones genistein and daidzein are thought to play a role in their putative health-enhancing properties such as prevention of certain cancers [1], decreased risk of cardiovascular diseases [2], and the improvement of bone health [3]. Soy isoflavones have also been reported to prevent growth of breast cancer cells [4,5].

An important role in this apparent controversy may be played by the two estrogen receptors (ER) that have been identified to date [6,7]. These receptors, derived from two different genes and referred to as ERα and ERβ, both bind to the consensus estrogen responsive element (ERE) enabling the transcription of an ERE-based reporter construct. The DNA-binding domains of ERα and ERβ show a high degree of homology but the ligand-binding domains show only 59% homology. In general, both receptors display similar ligand binding profiles, but phytoestrogens like genistein and coumestrol induce receptor-dependent transcription stronger with ERβ than with ERα [8,9]. Furthermore, the tissue distribution of ERα and ERβ also differs substantially [8,10]. ERβ is very important in the bone, urogenital tract, cardiovascular system, central nervous system and the developing brain [11,12]. ERα seems the more important receptor type in the mammary gland and the uterus. It has been hypothesised that ERβ is important for the protection against hyperproliferation and carcinogenesis in breast, prostate and the gastrointestinal tract [11,13,14,15,16,17,18]. These observations led to the hypothesis of potential differences in the biological function and tissue-selective actions of the two receptors. These observations also suggest the existence of two previously unrecognised pathways of estrogen signalling: via the ERβ subtype in tissues exclusively expressing this subtype and, since estrogen receptors are known to function as dimers [19], via the formation of heterodimers in tissues expressing both subtypes. The differences in tissue distribution may also be very important from a pharmaceutical point of view, as hormone replacement therapy in postmenopausal women is an increasingly significant health issue [20].

In order to investigate the properties of chemicals and the presence of hormonally active substances in food and water, it is important to have rapid, robust and high-throughput bioassays. The effects of estrogens on different tissues stress the need for bioassays with both estrogen receptor types. When sensitivity is required, like in cases of small sample volumes and/or low concentrations, mammalian assays can best be used as they have lower detection limits than yeast-based assays [21]. However, because of the differences observed in the ERα and ERβ activity in different cell lines [22,23], the differences in estrogenic activities of substances by both receptor types can as well be

studied in yeast estrogen transcription activation assays with a consensus ERE-reporter construct. Furthermore, toxicity of samples to yeast or mammalian cells is a potential problem in assessing estrogenic activity in complex samples. As cytotoxicity occurs more frequently in mammalian cell assays than in yeast assays [24,25] and because yeast is more resistant to environmental contaminants such as heavy metals and bacterial endotoxins [26], yeast assays can best be used to study the estrogenic activity in such samples.

Several different yeast assays have been described, all of them using plasmid-based constructs for either the receptor or reporter construct, and β-galactosidase as the reporter protein [9,27,28,29]. With the exception of Morito et al. [9] these assays only use the hERα receptor. Previously we described the construction of a rapid yeast bioassay, with constructs for the human estrogen receptor α (hERα) and the ERE-reporter construct stably integrated into the genome [30]. This ERE-reporter construct consists of two consensus EREs with a centre-to-centre spacing of 40 bp that was placed in a truncated CYC1 promoter. Furthermore, yeast enhanced green fluorescent protein (yEGFP) was used as reporter protein, allowing a much easier and more rapid detection of estrogenic activity. In the present study we describe the development and properties of a similar assay based on the stable expression of human estrogen receptor β (hERβ). In order to validate both assays, a large number of compounds with known estrogenic properties were tested. These include natural hormones and their conjugated forms and metabolites, synthetic hormones like ethynylestradiol, estradiolbenzoate and DES, phytoestrogens like genistein, coumestrol and daidzein, the mycotoxin zearalenone and its derivatives, and environmental pollutants like p-nonylphenol.

2. Materials and Methods

2.1 Chemicals

The following compounds were purchased from Sigma: 17β-estradiol (E2β, CASRN 50-28-2), 17α-estradiol (E2α, 57-91-0), 17β-estradiol 3-benzoate (E2-benz, 50-50-0), 17β-estradiol 3-sulfate (E2-sul, 4999-79-5), 17β-estradiol 3-(β-D-glucuronide) (E2-3-gluc; 14982-12-8), 17β-estradiol 17-(β-D-glucuronide) (E2-17-gluc, 15087-02-2), estrone (E1, 53-16-7), estrone 3-sulfate (E1-sul, 438-67-5), estrone β-D-glucuronide (E1-gluc, 15087-01-1), estriol (E3, 50-27-1), 17α-ethynylestradiol (EE2, 57-63-6), dienestrol (84-17-3), mestranol (72-33-3), medroxyprogesterone 17-acetate (MPA, 71-58-9), progesterone (P, 57-83-0), testosterone (T, 58-22-0), 19-nortestosterone (19-norT, 434-22-0), 2-deoxy-20-hydroxyecdysone (β-ecdysone, 17942-08-4), zearalenone (z-lenone, 17924-92-4), zearalanone (z-lanone, 5975-78-0), α-zearalenol (α-z-lenol, 36455-72-8), α-zearalanol (α-z-lanol, 26538-44-3), β-zearalenol (β-z-lenol, 71030-11-0), β-zearalanol (β-z-lanol, 42422-68-4), genistein (446-72-0), genistin (529-59-9) and daidzein (486-66-8). Daidzin (552-66-9) was obtained from Plantech (U.K.), diethylstilbestrol (DES, 56-53-1) from Interpharm (The Netherlands) and hexestrol (84-16-2) from ICN. Coumestrol (479-13-0), enterolactone (78473-71-9), enterodiol (80226-00-2) and 4-nonylphenol (NP, 84852-15-3/104-40-5) were obtained from Fluka and 4-n-nonylphenol (4nNP, 104-40-5) from Riedel-de Haën. Naringenin (Nar, 480-41-1) and 8-prenylnaringenin (8-prenylN) were purchased from Apin Chemical Limited (UK) and 2-hydroxyestradiol (2OH-E2, 362-05-0), 4-hydroxyestradiol (4OH-E2, 5976-61-4), 2-hydroxyestrone (2-OH-E1, 362-06-1) and 4-hydroxyestrone (4-OH-E1, 3131-23-5) were obtained from Steraloids (USA). From all these compounds fresh stock solutions were made in either ethanol or DMSO as indicated.

2.2 Yeast strain

The yeast Saccharomyces cerevisiae (CEN.PK 102-5B, K20, URA3-, HIS3-, LEU-) was a gift from H. Silljé (University of Utrecht).

2.3 Plasmids

For the expression of the human estrogen receptor α, the p403-GPD yeast expression vector was used. For the expression of the human estrogen receptor β, the p405-GPD yeast expression vector was used. For the construction of the reporter plasmid, the p406-CYC1 yeast expression vector was used. All three plasmids were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and are described by Mumberg et al. [31].

2.4 Construction of the p405-GPD-ERβ receptor expression vector

The construction of the p403-GPD-ERα receptor expression vector, that contains the HIS3 marker gene, is already described in Bovee et al. [30]. The p405-GPD-ERβ receptor expression vector, containing the LEU marker gene, is made in a similar way. Briefly: synthesis of cDNA was carried out on isolated mRNA of T47 D human breast cancer cells and of mRNA isolated from human intestinal Caco-2 cells. Full-length human estrogen receptor β cDNA was obtained by PCRs using the T47 D cDNA, marathon uterus cDNA (human, Clontech) and the human intestine cDNA with the Expand High Fidelity PCR System (Boehringer Mannheim). PCR was performed using the following conditions: 34.2 μl ultra pure water, 5 μl 25 mM MgCl2, 5 μl Expand HF 10x concentrated buffer (without MgCl2), 0.8 μl 25 mM dNTP mix, 1 μl of the enzyme mix, 2 μl of the different cDNAs and 2 μl of a primer mix containing 10 μM of each primer were pipetted into a thin-walled PCR tube and PCR was performed in an Eppendorf Mastercycler gradient using the following cycle profile: 1) denature template 3 min at 95°C; 2) denature template 30 s at 94°C; 3) anneal primers 1 min at 60°C; 4) elongation 2 min at 72°C; 5) go to step 2 and repeat 35 times; 5) elongation 7 min at 72°C and step 7) for ever 10°C. After this first PCR, a second PCR was performed with the same conditions as described above but now 2 μl of the first PCR mixture was used instead of 2 μl of the different cDNAs. The sequence of the 5'-primer was as follows: 5'-CGTCTAGAGCTGTTATCTCAAGACATGGATATAA-3' and this primer contains a restriction site for Xba I just before the ATG start codon. The sequence of the 3'-primer is as follows: 5'-TAGGATCCGTCACTGAGACTGTGGGTTCTG-3' and this primer contains a restriction site for BamH I just after the TGA stop codon. This PCR generated a full-length ds cDNA of 1626 bp containing the 1593 bp coding sequence of the human estrogen receptor β gene with a 5'-Xba I and a 3'-BamH I restriction site just outside the coding sequence. This full length ERβ PCR product was isolated from a 1% low-melt agarose gel and ligated into a pGEM-T Easy Vector (Promega). Plasmid digestion control revealed several good clones and good clones were sequenced in both directions using the SEQ 4x4 apparatus and the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit, all used according to the manufacturers instructions (Amersham Pharmacia). All 1593 bp, from the ATG start to the TGA stop, were compared with the estrogen receptor β sequence published by Ogawa et al. [32].

Compared to this sequence, the ERβ cDNA clone obtained from the breast cells contained two mutations. The bp at position #430 consisted of T instead of A and #1030 consisted of G instead of A. Both mutations resulted in changed codons that will change the amino acid translated from it, respectively Arginine to Tryptophan and from Methionine into Valine. Probably this clone also misses a G at bp position #216. This clone was therefore not used. The ERβ cDNA clone obtained from human uterus contained five mutations. The bp at position #349 consisted of C instead of T, #601 C instead of T, #800 G instead of A, #965 C instead of T and #1245 consisted of G instead of A. The mutations at bp positions #349 and #1245 result in changed codons, but these changed codons will be translated into the same amino acids as the original codons. However, the mutations at bp positions #601, #800 and #965 result in changed codons that also change the amino acid that will be translated from it, respectively Cysteine to Arginine, Glutamine to Arginine and from Leucine to Proline. This clone was therefore not used. The ERβ cDNA clone obtained from human intestine contained 7 A nucleotides instead of 6 A nucleotides at bp position #9 to #14. This will result in a frame shift that changes the entire sequence and therefore this clone was not used.

To obtain a cDNA clone that is fully complementary to the sequence published by Ogawa et al. [32], the Xba I - Msc I part from this intestine ERβ cDNA clone, containing the 7 A instead of 6 A, was replaced by the Xba I - Msc I part of the uterus ERβ cDNA clone. Although the uterus ERβ cDNA clone contained five mutations, the first mistake is at bp position #349 and so the Xba I - Msc I part of this clone does not contain any mistake. In this way a human ERβ cDNA clone was obtained that fully corresponded to the estrogen receptor β sequence published by Ogawa et al. [32]. This uterus/intestine human ERβ cDNA was cut out of the pGEM-T Easy plasmid with Xba I and BamH I and cloned into the corresponding Xba I - BamH I site of the p405-GPD expression vector. This p405-GPD-ERβ vector was used to transform Epicurian Coli XL-2 Blue Cells. Plasmid digestion controls and PCR controls of single white colonies were performed and revealed several good clones (data not shown).

2.5 Construction of yeast hERα and hERβ cytosensors

The yeast cytosensor expressing the hERα is the one already described in Bovee et al. [30]. The yeast cytosensor expressing the hERβ and the yeast cytosensor that expresses both ERα and ERβ are made in a similar way. Briefly, construction of yeast hERα and hERβ cytosensors was started with the stable transformation of yeast K20 (Ura-, His- and Leu-) with the p406-ERE2s2-CYC1-yEGFP

reporter vector, integrated at the chromosomal location of the Uracil gene via homologous recombination. Transformants were grown on MM/LH plates and PCR and Southern blot hybridisation were used to select clones in which the integration has occurred at the desired URA3 site with only a single copy of this reporter vector. Subsequently, this yeast reporter strain was stably transformed with the p403-GPD-ERα, the p405-GPD-ERβ or both expression vectors and transformants were grown respectively on MM/L, MM/H or MM plates. Actually, the cytosensor containing both receptor types was made by transforming the ERα cytosensor with the p405-GPD-ERβ receptor expression vector.

2.6 Yeast culturing conditions

Before running an assay, an agar plate containing the selective MM/L, MM/H or MM medium was inoculated with respectively the yeast ERα, ERβ or ERα/β cytosensor from a frozen -80°C stock (20% glycerol v/v). The plate was incubated at 30°C for 24-48 h and then stored at 4°C. The day before running the assay, a single colony of the yeast cytosensor was used to inoculate 10 ml of the corresponding selective medium. This culture was grown overnight at 30°C with vigorous orbital shaking at 225 rpm. At the late log phase the yeast ERα cytosensor culture was diluted (1:10) in MM/L, the yeast ERβ cytosensor was diluted (1:20) in MM/H and the yeast ERα/β cytosensor was diluted (1:20) in MM. This minimal medium (MM) consisted of yeast nitrogen base without amino acids or ammonium sulphate (1.7 g/l), dextrose (20 g/l) and ammonium sulphate (5 g/l). The MM/L and MM/H medium were supplemented with respectively L-leucine (60 mg/l) or L-histidine (2 mg/l).

2.7 yEGFP assay: exposure to different substances and the measurement of estrogenic activities

For exposure in 96 well plates (Costar), aliquots of 200 μl of the yeast culture were pipetted into each well. Exposures to different doses of 17β-estradiol and all the other substances were performed through the addition of 1 μl of an ethanol or DMSO stock solution to each well, resulting in 0.5% final concentration of the solvent. Ethanol and DMSO only controls were included in each experiment and each sample concentration was assayed in triplicate. Exposures were performed for 4 and 24 hours. Fluorescence at these time intervals was measured directly in the CytoFluor Multi-Well Plate Reader (Series 4000, PerSeptive Biosystems) using excitation at 485 nm and measuring emission at 530 nm. The densities of the yeast culture at these time intervals were also determined by measuring the OD at 630 nm. This was done to check whether a substance was toxic for yeast. If there were no differences in yeast densities, the fluorescence signals were corrected with the signals obtained with the blank medium (supplemented MM containing 0.5% ethanol or DMSO solvent only).

3. Results

Recombinant yeast cells were constructed that either express the human estrogen receptor α, β or both, and yEGFP in response to exposure to estrogens. All constructs, both the receptor construct as well as the reporter constructs, were stably integrated into the yeast genome by the use of yeast integrating plasmids The construction of the yeast cell expressing the hERα was described previously, including that of the cDNA encoding for the alpha receptor [30]. This cDNA, constructed from mRNA of T47D cells, had the same sequence as described by Greene et al. [33]. The construction of cDNA for the hERβ-receptor was initially attempted from the mRNAs isolated from T47D human breast cancer cells, human intestinal Caco-2 cells and from human uterus cDNA. Since all three cDNAs contained mutations in comparison to the sequence published by Ogawa et al. [32], the eventual cDNA introduced into the yeast cytosensor was constructed from the latter two cDNAs (see section 2.4).

A number of compounds with known estrogenic properties were tested in the ERα-assay. As shown in Figure 1, compounds like DES, ethynylestradiol, and genistein all caused a dose-related increase in the production of green fluorescent protein after a relatively short exposure period of 4 h. Dose-response curves were similar after 24 h but in general allowed a better curve-fit (Figures 2A and 2B).

Figure 3 shows the dose-response curve for 17β-estradiol obtained after 4 h with the yeast cytosensors expressing hERα, hERβ or both the hERα and hERβ receptors. The maximal transcriptional activity of the ERβ cytosensor is only about 40% of the maximal activity observed with the ERα cytosensor, but the ERβ cytosensor showed a higher response at lower concentrations. This is reflected in the much lower EC50 for 17β-estradiol, the concentration where half-maximal activation is reached, being respectively 0.06 and 0.6 nM for the ERβ and ERα cytosensor. In contrast to the ERα cytosensor, the dose-response curves obtained with the ERβ and the ERα\ERβ cytosensors after a 24 h exposure period were very poor (data not shown).

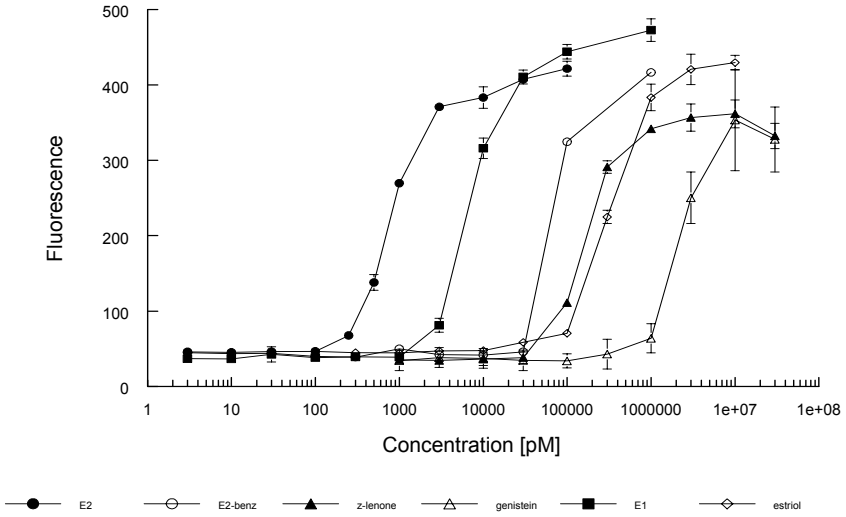

Figure 1. Response of the yeast ERα cytosensor to different substances after a 4 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 µl of a yeast culture of the ERα cytosensor, an aliquot of 1 µl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Table 1 and 2. Fluorescence was determined after 4 hours as described in Materials and Methods (see section 2.7). Cells were exposed to 17β-estradiol (E2), β-estradiol 3-benzoate (E2-benz), zearalenone (z-lenone), genistein, estrone (E1), or estriol. Fluorescence signals are the mean of a triplicate with sd.

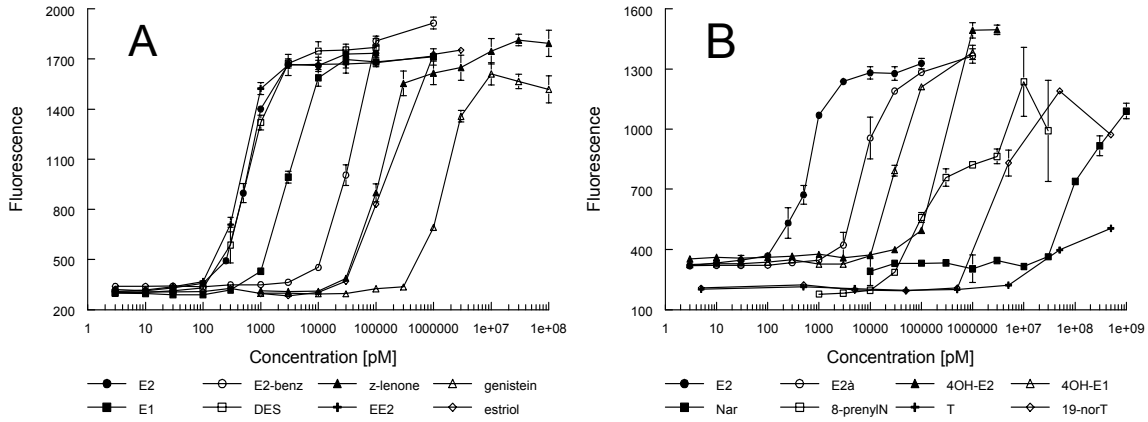

Figure 2. Response of the yeast ERα cytosensor to different substances after a 24 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 µl of a yeast culture of the ERα cytosensor, an aliquot of 1 µl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Table 1 and 2. Fluorescence was determined after 24 hours as described in Materials and Methods (see section 2.7). Figure 2A shows dose-response curves of 17β-estradiol (E2), β-estradiol 3-benzoate (E2-benz), zearalenone (z-lenone), genistein, estrone (E1), diethylstilbestrol (DES), 17α-ethynylestradiol (EE2) and estriol and figure 2B shows dose-response curves of 17β-estradiol (E2), 17α-estradiol (E2α), 4-hydroxyestradiol (4OH-E2), 4-hydroxyestrone (4OH-E1), naringenin (Nar), 8-prenylnaringenin (8-prenylN), testosterone (T) and 19-nortestosterone (19-norT). Fluorescence signals are the mean of a triplicate with sd.

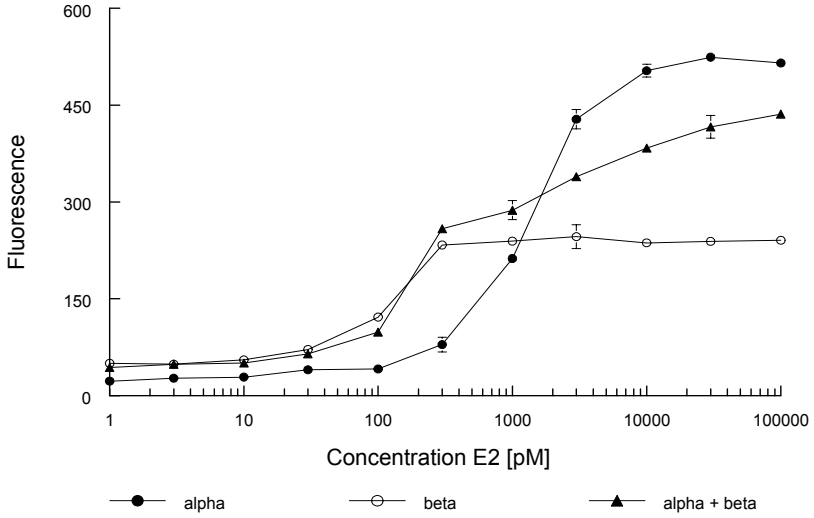

Figure 3. Response of the yeast ERα cytosensor, ERβ cytosensor and the yeast cytosensor that expresses both the ERα and the ERβ after exposures for 4 h to 17β-estradiol (E2). Exposure was started by adding to 200 µl yeast culture of the corresponding cytosensors, an aliquot of 1 µl of an E2 stock solution in DMSO. Fluorescence was determined after 4 h as described in Materials and Methods (see section 2.7) and fluorescence signals are the mean of a triplicate with sd.

Figures 4A and 4B show dose-response curves obtained with the ERβ cytosensor after a 4 h exposure to a number of different compounds. Table 1 shows the calculated EC50 values for 17β-estradiol, a number of metabolites and other hormonal substances, as obtained by a mathematical non-linear regression curve-fit formula (y=a0+a1/(1+(x/a2)^a3)). The relative estrogenic potencies (REP) of these substances, defined as the ratio between the EC50 of 17β-estradiol and the EC50 of the compound, with both the ERα and the ERβ cytosensor are also shown in Table 1. Figure 5 presents a graphical comparison of these EC50 values. These data show that e.g. estrone, the main metabolite of 17β-estradiol, showed a REP of 0.2 with ERα and 0.1 with ERβ. Other metabolites of E2β, like the hydroxy metabolites and the sulfate and glucuronide conjugates, are much less potent than E2β itself, displaying relative potencies of less than 0.05. However, the hydroxy-metabolites are more potent than the conjugated forms. There are only small differences between the relative potencies with ERα and ERβ of these natural hormones and their conjugated forms and metabolites. However, like estrone, the conjugated forms (estrone 3-sulfate and estrone β-D-glucuronide) and metabolites (2-hydroxyestrone and 4-hydroxyestrone) are slightly more potent with ERα than they are with ERβ (see Figure 5). The REP with ERα and ERβ of 2-OH-E1 are respectively 0.0026 and 0.00026, of 4-OH-E1 respectively 0.022 and 0.0048, of E1-3-sulfate respectively 5 x 10-5 and 1.1 x 10-5, and of E1-β-D-glucuronide respectively <1 x 10-5 and 4 x 10-6. The differences with the synthetic hormones dienestrol, hexestrol, mestranol and DES are more obvious. Dienestrol, hexestrol and especially mestranol are relatively much more potent with the ERα, showing REPs of respectively 0.56, 0.36 and 0.11, than with the ERβ, showing REPs of respectively 0.091, 0.091 and 0.0001. DES on the other hand showed a two-fold higher potency with the ERβ than with the ERα (see Figure 5).

Table 2 shows the EC50 values and REPs for the phytoestrogens and resorcyclic acid lactones. Based upon these data and the dose-response curves, the ranking of the relative estrogenic potencies of the phytoestrogens and resorcyclic acids with the ERα is as follows: 17β-estradiol >> 8-prenylnaringenin > coumestrol > zearalenone >> genistein >> genistin > naringenin. For ERβ, the ranking is as follows: 17β-estradiol >> coumestrol > genistein > zearalenone > 8-prenylnaringenin >> daidzein > naringenin > genistin >> daidzin. For the phytoestrogens REP values with the ERβ were in general higher than with the ERα, with the exception of 8-prenylnaringenin (see Figure 5).

Nearly all compounds were tested twice and some of them even three times. The differences in the determined EC50 and REP values were very small and negligible (data not shown). The variation in the determined EC50 values for the 17β-estradiol reference (see table 1 and 2) is mainly due to the solvent used, either ethanol or DMSO [30]. The variation due to inter-experimental differences is less important. To correct for inter-experimental differences and the influence of the solvent used, each new experiment has its own 17β-estradiol reference and compounds dissolved in ethanol or DMSO are compared with a estradiol reference dissolved in the same solvent.

Table 1. EC50 concentration and relative estrogenic potency (REP) of compounds with ERα and ERβ

Compound	EC50[b] [nM] ERα	REP[c] ERα	EC50[b] [nM] ERβ	REP[c] ERβ
17β-estradiol	0.5-1.0[d]	1.0	0.06-0.25[d]	1.0
17α-ethynylestradiol	0.5	1.2	0.12	1.0
Diethylstilbestrol	0.6	1.0	0.06	2.0
β-estradiol 3-benzoate	70	8.6 E-3	30	8.3 E-3
Hexestrol[a]	2.8	0.36	1.1	9.1 E-2
Dienestrol[a]	1.8	0.56	1.1	9.1 E-2
Mestranol[a]	9.1	0.11	1.0 E2	1.0 E-4
Medroxyprogesterone 17-acetate[a]	n.r.	n.r.	n.r.	n.r.
Progesterone[a]	n.r.	n.r.	n.r.	n.r.
Testosterone[a]	>3 E4[e]	<3 E-5[e]	n.r.	n.r.
19-nortestosterone[a]	3.0 E3	2.8 E-4	>5 E4	<1.7 E-6
Estrone	3	0.2	1.1	0.1
Estriol	1.2 E2	5.0 E-3	12	5.0 E-3
17α-estradiol	7	9.3 E-2	2.8	2.1 E-2
2-hydroxyestradiol	60	1.1 E-2	8.5	7.1 E-3
4-hydroxyestradiol	2.5 E2	2.6 E-3	20	3.0 E-3
2-hydroxyestrone	2.5 E2	2.6 E-3	2.3 E2	2.6 E-4
4-hydroxyestrone	30	2.2 E-2	12.5	4.8 E-3
β-estradiol 3-sulfate[a]	3.4 E3	2.6 E-4	4.0 E2	2.3 E-4
β-estradiol 3-β-D-glucuronide[a]	3.8 E3	2.4 E-4	1.8 E2	5.0 E-4
β-estradiol 17-β-D-glucuronide[a]	>4.0 E4	<2.0 E-5	>8.0 E2	<1.1 E-4
Estrone 3-sulfate[a]	1.8 E4	5.0 E-5	8.0 E3	1.1 E-5
Estrone β-D-glucuronide[a]	>8 E4	<1 E-5	>2.8 E4	<4 E-6

n.r. = no response

[a] For these compounds DMSO is used as solvent (all the other compounds are tested in EtOH as solvent).

[b] The EC50 is the concentration giving a half-maximum response. Exposure was 24 h and 4 h for the ERα and ERβ cytosensor respectively.

[c] The relative estrogenic potency (REP) is defined as the ratio between the EC50 of E2β and the EC50 of the compound.

[d] The range in EC50 values of 17β-Estradiol. E2β is tested in DMSO and in EtOH as solvent and the EC50 for E2β is lower in EtOH as solvent than it is in DMSO [30]. There are also small differences in the EC50 values between the different exposures, as all described compounds are not tested in one experiment, but in different exposures during a period of about six months. Here the range of the obtained EC50 values is given.

[e] The greater than (>) sign means that this compound does not fully reach a maximum response and therefore the EC50 value cannot be determined accurately. The real EC50 will be greater than the value that is given in the table. As a result REPs are indicated with the smaller than sign (<).

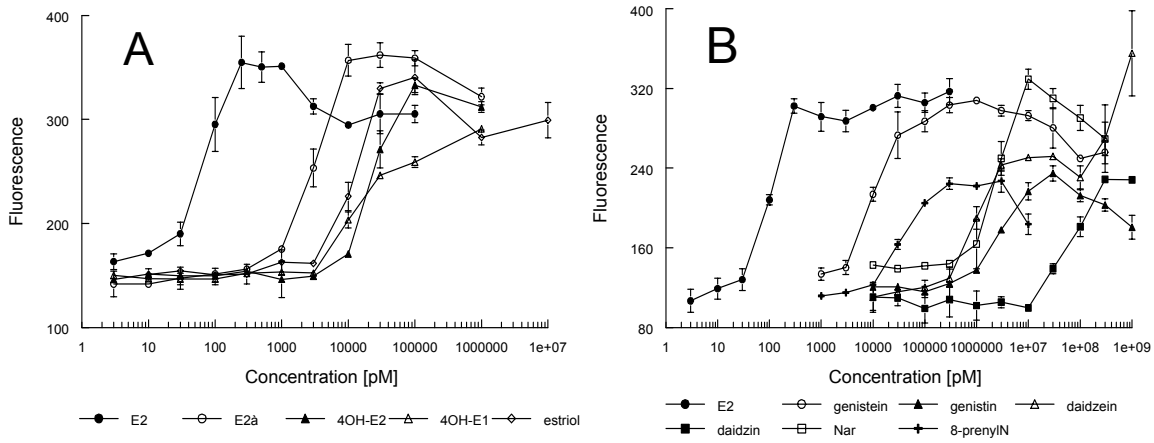

Figure 4. Response of the yeast ERβ cytosensor to different substances after a 4 h exposure period. Exposure to 17β-estradiol and other substances was started by adding to 200 μl of a yeast culture of the ERβ cytosensor, an aliquot of 1 μl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Table 1 and 2. Fluorescence was determined after 4 hours as described in Materials and Methods (see section 2.7). Figure 4A shows dose-response curves of 17β-estradiol (E2), 17α-estradiol (E2α), 4-hydroxyestradiol (4OH-E2), 4-hydroxyestrone (4OH-E1) and estriol and figure 4B shows dose-response curves of 17β-estradiol (E2), genistein, genistin, daidzein, daidzin, naringenin (Nar) and 8-prenylnaringenin (8-prenylN). Fluorescence signals are the mean of a triplicate with sd.

4. Discussion

The present paper demonstrates the successful construction of yeast cells stably expressing the human estrogen receptors α and β, and producing green fluorescent protein in response to compounds with known estrogenic properties. In addition to the biomolecular controls, this is clearly shown by the very reproducible dose-related transcription activation of the yEGFP gene in both yeast ERα and ERβ cytosensors after exposure to 17β-estradiol (Figures 1-4). The poor dose-response curves obtained with the ERβ and ERα/ERβ cytosensors after 24 h exposure indicates that the ERβ receptor protein is not as stable as the ERα or that a complex with the ERβ receptor is involved in a quicker signalling pathway.

One of the potential applications of the cytosensors is their use for detecting increased hormonal activity in samples of illegally treated animals. Therefore it is essential to obtain data on the estrogenic potency of known and putative metabolites and to show the specificity of the assay for estrogenic compounds. The data in Table 1 show that the cytosensors are specific for estrogens, since the gestagens progesterone and medroxyprogesterone 17-acetate, and the androgen testosterone did not show a clear response. Only 19-nortestosterone gave a full dose-response curve, but this compound appears to have both androgenic and estrogenic properties, possibly explaining its excellent anabolic properties requiring both types of hormonal activity. Here we show for the first time that 19-nortestosterone is a full agonist with ERα and that this compound is relatively more potent with ERα than it is with ERβ (Table 1, Figure 5). A number of metabolites of 17β-estradiol and estrone showed a clear response, but in general at least a factor 5 to 105 less then their parent compounds. There are only small differences between the relative potencies with ERα and ERβ of the natural hormones and their conjugated forms and metabolites. However, like estrone, the conjugated forms E1-3-sulfate and E1-β-D-glucuronide and metabolites 2-OH-E1 and 4-OH-E1 appear to be slightly more potent with ERα than with ERβ. The glucuronidated forms of 17β-estradiol, E2β-3-β-D-glucuronide and E2β-17-β-D-glucuronide, on the other hand seem slightly more potent with ERβ (see Figure 5) and 17α-estradiol is more potent with ERα. The present data also show that for testing of e.g. urine of calves a deconjugation step is a requirement for gaining sensitivity. As 1% deconjugation of the 17β-estradiol conjugates would already result in a REP of 0.01 and as the REPs of these

conjugates are much lower than 0.01 and because dose-response curves after a relatively short exposure period of 4 h were similar to those after 24 h, it is most likely that yeast is not able to deconjugate these compounds. For the same reasons the estrogenic activity of β-estradiol 3-benzoate, REP of 8.6 E-3, is probably not due to metabolic conversion of the ester. In the present study, no attempts were made to investigate the metabolism of the test compounds by the cells, which may result in activation or deactivation of compounds and may explain possible differences in estrogenic potencies with other test systems [8,9,21-29].

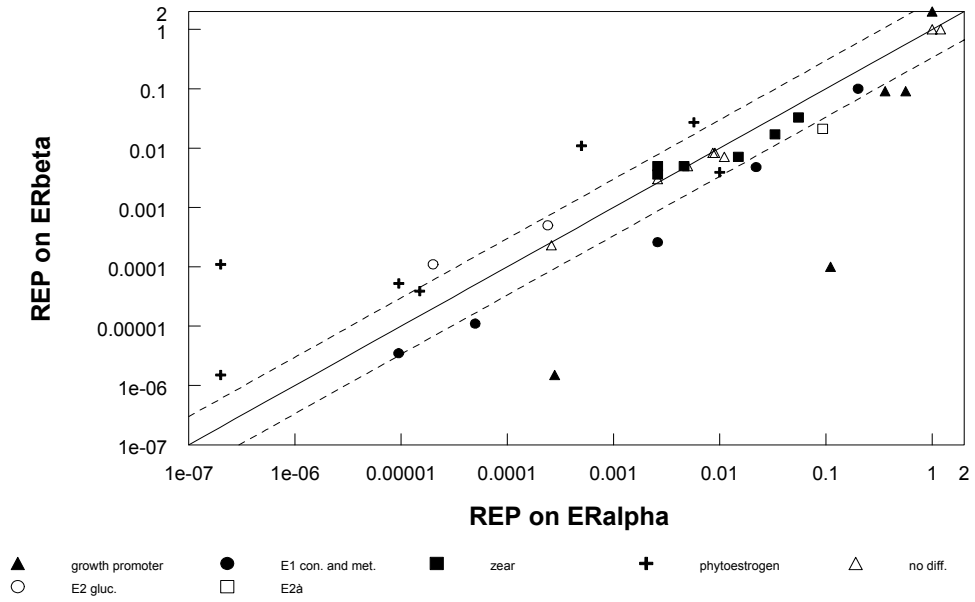

Figure 5. Relative Estrogenic Potencies (REP) of all compounds with the ERα and ERβ cytosensor. Exposure to 17β-estradiol and other substances was started by adding to 200 µl of a yeast culture of the corresponding cytosensors, an aliquot of 1 µl of a stock solution, using either ethanol or DMSO as a solvent as indicated in Table 1 and 2. Fluorescence was determined after 4 or 24 h as described in Materials and Methods (see section 2.7) and as indicated in Table 1 and 2. The growth promoters are: DES, hexestrol, dienestrol, mestranol and 19-norT; E1 con. and met. are: E1, 2OH-E1, 4OH-E1, E-3-sulfate and E1-β-D-glucuronide; zear are: z-lenone, z-lanone, α-z-lenol, α-z-lanol, β-z-lenol and β-z-lanol; phytoestrogen are: coumestrol, genistein, genistin, daidzein, daidzin, nar and 8-prenylN; no diff. are: E2β, EE2, E2-benz, E3, 2OH-E2, 4OH-E2, E2-sul and NP and the E2-gluc. are : E2-3-gluc and E2-17-gluc. The line x=y and the dotted lines x=3y and 3x=y are also shown. Compounds between the lines have more or less the same activity with ERα and ERβ, whereas compounds above the upper dotted line are three times or more active with ERβ than with ERα. Compounds below the lower dotted line are three times or more active with ERα than with ERβ.

A specific difference in the ligand-binding properties of the two receptors is the affinity for phytoestrogens [8,9]. As described previously, the isoflavones genistein, genistin, daidzein and daidzin, the coumestran coumestrol and the flavonoid naringenin were relatively more potent with ERβ than with ERα (see Table 2 and Figure 5). Coumestrol and genistein were by far the most potent of these compounds with ERβ. However, 8-prenylnaringenin, a phytoestrogen present in hops, was relatively more potent with ERα than with ERβ and was actually the most potent phytoestrogen with ERα. Until now, comparison of 8-prenylnaringenin with ERα and ERβ was only performed in a receptor binding assay [34], revealing comparable binding activity to both receptor forms. Here we describe for the first time, that 8-prenylnaringen is more potent with ERα than with ERβ, using a yeast transcription activation assay. Although the data obtained from transcription activation assays are in general comparable with results from radioligand competition binding assays, Kuiper et al. also

observed that although coumestrol bound to the ERβ with the same affinity as 17β-estradiol, transcription activation started at 1000 times higher concentrations for coumestrol [8]. Furthermore, ligand-binding assays do not disclose the biological activity of a compound, i.e. whether it is an agonist or an antagonist and therefore, transcription activation assays are supposed to correspond better with effects found in vivo. The characteristics of 8-prenylnaringenin, being a very potent compound that is relatively more potent with ERα than with ERβ, are in agreement with effects described upon the intake of the female flowers of the hop plant, as they have long been used as a preservative. More recently they have also been included in some herbal preparations for women for breast enhancement [34,35]. Both applications indicate that 8-prenylnaringenin is an active agonist with ERα. Naringenin, a flavonoid present in citrus fruits, is relatively more potent with ERβ, but compared to 8-prenylnaringenin, this compound is only a weak estrogen. The lignans enterolactone and enterodiol did not show any response with both receptor types and are therefore characterised as being nonestrogenic in our yeast cytosensors.

Table 2. EC50 concentration and relative estrogenic potency (REP) of resorcyclic acid lactones, phytoestrogens, natural compounds and nonylphenol with ERα and ERβ

Compound	EC50[b] [nM] ERα	REP[c] ERα	EC50[b] [nM] ERβ	REP[c] ERβ
17β-estradiol	0.5-1.0[d]	1.0	0.06-0.25[d]	1.0
Zearalenone	1.3 E2	4.6 E-3	20	5.0 E-3
Zearalanone	40	1.5 E-2	14	7.1 E-3
α-zearalenol	11	5.5 E-2	3	3.3 E-2
α-zearalanol	18	3.3 E-2	6	1.7 E-2
β-zearalenol	2.3 E2	2.6 E-3	28	3.6 E-3
β-zearalanol	2.3 E2	2.6 E-3	20	5.0 E-3
Coumestrol[a]	1.4 E2	5.7 E-3	3	2.7 E-2
Genistein[a]	2.0 E3	5.0 E-4	8	1.1 E-2
Genistin[a]	>4 E4[g]	<2 E-5[g]	2.3 E3	3.9 E-5
Daidzein[a]	n.r.	n.r.	8.0 E2	1.1 E-4
Daidzin[a]	n.r.	n.r.	6.0 E4	1.5 E-6
Enterolactone[a]	n.r.	n.r.	n.r.	n.r.
Enterodiol[a]	n.r.	n.r.	n.r.	n.r.
ß-ecdysone[a]	n.r.	n.r.	n.r.	n.r.
Naringenin[a]	>7 E4	<1 E-5	2.1 E3	5.2 E-5
8-prenylnaringenin[a]	1.0 E2	1.0 E-2	33	3.9 E-3
4-n-nonylphenol[a,e]	n.r.	n.r.	n.r.	n.r.
4-nonylphenol[a,f]	1.0 E2	9.0 E-3	30	8.3 E-3

n.r. = no response

[a] For these compounds DMSO is used as solvent (all the other compounds are tested in EtOH as solvent).

[b] The EC50 is the concentration giving a half-maximum response. Exposure was 24 h and 4 h for the ERα and ERβ cytosensor respectively.

[c] The relative estrogenic potency (REP) is defined as the ratio between the EC50 of E2β and the EC50 of the compound.

[d] The range in EC50 values of 17β-Estradiol. E2β is tested in DMSO and in EtOH as solvent and the EC50 for E2β is lower in EtOH as solvent than it is in DMSO [30]. There are also small differences in the EC50 values between the different exposures, as all described compounds are not tested in one experiment, but in different exposures during a period of about six months. Here the range of the obtained EC50 values is given.

[e] Pure 4-n-nonylphenol obtained from Riedel-de-Haën.

[f] Technical mixture of p-isomers of 4-nonylphenol obtained from Fluka.

[g] The greater than (>) sign means that this compound does not fully reach a maximum response and therefore the EC50 value cannot be determined accurately. The real EC50 will be greater than the value that is given in the table. As a result REPs are indicated with the smaller than sign (<).

There are no great differences between the relative potencies of zearalenone and its derivatives with both receptor types (Table 2, Figure 5). However, compared to the natural phytoestrogens, zearalenone is about as potent as the most potent phytoestrogens coumestrol, genistein and 8-prenylnaringenin, whereas the derivatives α-zearalenol and α-zearalanol are even more potent. The latter is not unexpected, since α-zearalanol is used as a legal growth promoter for cattle breeding in the US [36]. Very similar REPs for this series of resorcyclic acid lactones have been reported by Le Guevel and Pakdel [37], also using a yeast assay with hERα.

Recently, ICCVAM [38] included the environmental pollutant 4-n-nonylphenol [CASRN 104-40-5] as a positive control in a set of reference compounds for transcriptional activation assays. In our hands this compound did not show a response in neither of the yeast cytosensors. Others however, have reported 4-n-nonylphenol to be active in these type of assays [39,40]. Thorough review of these studies showed that a technical mixture like the one available from Fluka (approximately 85 to 92.7% of branched isomers) or p-nonylphenol (CAS No. 84852-15-3) were used instead of the unbranched nonyl chain. When the 4-nonylphenol technical mixture of Fluka was used, it also showed a dose-response curve in our test with an EC50 of 100 nM (see table 1). Chemical analysis with GC/MS showed that there was actually no 4-n-nonylphenol, the aliphatic straight chain, in this technical mixture of Fluka (data not shown). From this, it can be concluded that 4-n-nonylphenol is not estrogenic and that the estrogenicity of the technical mixture is due to one or more isomers with a branched side-chain. A similar conclusion was presented by Pedersen et al. (1999) studying the induction of plasma vitellogenin in rainbow trout by linear and technical nonyl- and octylphenol [41].

The relatively very high estrogenic potencies of estrone and 4-hydroxyestradiol, as observed in the ER-CALUX assay with T47D cells, giving rise to REPs of respectively 1.0 and 0.45 [42], were not observed with our ERα cytosensor, in which REPs of respectively, 0.2 and 0.0026 were observed. This possibly points to important differences between yeast cells and mammalian cells, in for example the metabolism and absorption of compounds and the transcription activation pathway. However, as shown in the case of biochanin A and estrone for metabolism [8,42] and for transcription activation [22,23], similar differences exist between different mammalian cell lines. This yeast assay therefore represents another appropriate assessment of the relative activity of various estrogens.

In general the response obtained for E2β with the ERα receptor is in a similar dose-range as described for yeast by other authors [9,27,37,40]. The lower maximum response for ERβ, as compared to ERα, has been reported for mammalian cells as well [22,23], but the observation that the yeast cells expressing ERβ show a response at lower concentrations of 17β-estradiol has not been observed previously. Morito et al. [9] used a yeast estrogen assay and found that the dose-response curve obtained with 17β-estradiol for the yeast expressing the ERα was similar to the one obtained with the yeast expressing the ERβ. In Chinese hamster ovary cells (CHO) transiently transfected with an ERα or ERβ expression construct and an ERE-based reporter construct, the transcriptional activity after exposure to 17β-estradiol of ERβ was only about 50% of the activity observed with ERα. However, in this study with CHO cells, half-maximal activation was reached at lower concentrations for ERα than for ERβ, giving EC50 values for 17β-estradiol of 0.04 and 0.3 nM respectively [7,22]. At the same time, this latter study also revealed that the ability of ERβ to function as an estrogen-dependent transcriptional activator is highly dependent on the cellular context. It was shown that in human endometrial cancer (HEC-1) cells and MDA-231 breast cancer cells transcription activation after exposure to 17β-estradiol by ERβ was respectively only about 15% and less than 5% of the transcriptional activity by ERα. In this case, half-maximal activation was again reached at lower concentrations for ERα than for ERβ. Similar results were obtained with human embryonal kidney 293 cells.

Another interesting observation is the apparent lower response in the cells expressing both receptor types (Figure 3), indicating that either ERβ is dominating in the possible heterodimers or that ERβ prevents the formation or activity of the ERα homodimer. Thus, just like ER(beta)cx [17], it seems that ERβ is a dominant repressor of ERα function, at least in the case of yeast. These differences between both receptor types might reflect characteristics of the ERβ and support the hypothesis that ERβ may have a role in protection against hyperproliferation and carcinogenesis. Therefore, the repressor (ERβ) has to be more sensitive to 17β-estradiol, explaining the lower EC50 for ERβ than for ERα, but the maximum response should be lower, repressing the activity of the ERα. Even a lower stability of the repressor (ERβ) could support this hypothesis, as repressor activity is not needed all the time in a cell. Also, the inability to obtain a full length cDNA of the ERβ without any mutations (see section 2.4) from mRNA isolated from the human T47D breast cancer and Caco-2 colon cancer cells, might point at a contribution of an inactivated ERβ function, to the origin of the cancer. At the same

time this observation stresses the need to reinvestigate the identity and functionality of the estrogen receptors in the different bioassays used for testing estrogenic activity.

In conclusion, both the ERα cytosensor and ERβ cytosensor show clear dose-response curves when exposed to estrogenic compounds. These yEGFP assays are not only very sensitive, as shown by EC50 values for E2β of 0.5 and 0.06 nM for the ERα and ERβ cytosensor respectively, but are also very rapid, convenient, reproducible and most likely more robust than cell-lines. Both cytosensors can be used to study the estrogenicity of different compounds in order to determine the relative estrogenic potency of these compounds. Since good dose-response curves can be obtained after only 4 hours of exposure, the often questioned permeability of the yeast cell wall does not seem to be an obstacle in our yeast estrogen assay.

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5

The German bakery waste incident; use of a combined approach of screening and confirmation for dioxins in feed and food

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Abstract

During the last six years several incidents have occurred with dioxins in feed, stressing the need for rapid screening methods for these compounds. The most recent incident was the contamination of bakery waste used for animal feed due to the use of waste wood for drying of the material. In addition to Germany, the material was also shipped to the Netherlands. Levels up to 12 ng TEQ/kg have been detected, being about 15 times over the current limit of 0.75 ng TEQ/kg. In the Netherlands a combined strategy of screening with the CALUX-bioassay and the HRGC/HRMS confirmatory method was used to rapidly control the incident. Pigs were contaminated by the incident but only to a very limited extent. Despite the rather low limits for pig meat, the CALUX bioassay showed excellent performance, once again confirming the value of this assay.

1. Introduction

Dioxins have caused major problems in feed and food during the last decades. This started with the contamination of chicken feed during the sixties, as a result of the use of fat drippings from cowhides that had been treated with polychlorophenols. Millions of chickens died from a disease called chicken edema disease [1,2,3,4]. Contamination of rice oil for human consumption with PCB oil, containing also dioxins, caused major health effects in Taiwan and Japan [5,6]. Following the discovery in the late seventies that dioxins were also formed during waste incineration [7], it was shown that cows grazing in the vicinity of municipal waste incinerators produced milk with elevated dioxin levels [8]. This source was eliminated by adaptation of the incinerators and this was followed by a relatively quiet period. During this period, toxicological studies confirmed the high toxicity of these compounds and the original limit of 10 pg TEQ/kg body weight/day, based on tumour formation, was further lowered based on effects on the immune and reproductive system, as well as neurobehaviourial and neurodevelopmental effects. It is also evident that a number of PCBs, the so-called non- and mono-ortho PCBs can adapt a planar structure and cause similar effects as dioxins [9].

During the last six years several incidents with dioxins occurred in Europe, starting with contaminated feed and resulting in contaminated food for human consumption. This was actually preceded by the discovery in the US in 1998 that ball clay used for feed for catfish and poultry was contained high levels of dioxins resulting in contaminated fish and chickens [10,11]. Also in 1998, Malisch detected an increase of dioxin levels in milk and traced this back to the use of contaminated citrus pulp from Brazil [12]. The pulp had been mixed with contaminated lime, being a waste product from a PVC production plant. The incident also had a major impact in the Netherlands where milk levels increased up to 3-fold. A much greater impact had the use of PCB contaminated fat for the production of chicken and pig feed in the West of Belgium, resulting in very high levels in eggs and meat [13,14,15]. Since the contamination was only discovered after several months, most of the products had been consumed. Furthermore, the contamination could spread through the recycling of contaminated slaughterhouse offal. Nevertheless a massive control of products before consumption had to be carried out. The intensified analysis of samples resulted in the same year in the discovery of contaminated kaolinic clay, used for animal feed. In 2002 contaminated cholin chloride was discovered resulting from the use of pentachlorophenol contaminated wood chips as a carrier [16]. In particular the Belgium incident resulted in an increased effort of the EU to control the various sources and to limit the introduction of dioxins into the food chain. Among others, strict limits were set for feed and food [17,18].

During these various incidents it became apparent that especially during incidents but also for routine analysis, the use of rapid and cheap screening methods is an absolute necessity. In the past the so-called EROD-assay has been used, based on the increased expression of cytochrome P450 enzyme in liver cells exposed to dioxins. In the Netherlands, an alternative assay was developed, the CALUX-bioassay, based on an increased production of luciferase after exposure of mouse or rat hepatoma cells [19]. This assay was validated for milk fat [20] and used for the first time for feed during the citrus pulp incident and proved its value in subsequent years [21]. However, at present its use for routine screening in other countries is still limited, partly due to the absence of convincing data from international validation studies.

In February 2003, a rapid alert was issued by the EU, reporting the presence of elevated dioxin levels in dried German bakery waste used in animal feed. Levels reported varies between 13.3 ng TEQ/kg as measured by the Fresenius Institute in Bayreuth, and 3.3, 5.6 en 5.9 ng WHO TEQ/kg as measured by Food GMBH in Jena. Part of this bakery waste was used by a Dutch company for the

production of different types of feed. The source of the dioxins was reported to be the use of waste wood for the drying of the bakery waste. Based on the relative amounts of bakery waste used for the feed, it was shown that only in the case of pigs and calves there was a chance that the EU limits for animal products could be exceeded. Intensive tracking and tracing was performed by the Dutch Inspection Services resulting in over 300 samples of bread meal, feed and calf and pig fat. All samples were analysed with CALUX. Suspected samples and part of the negative samples were investigated by GC/MS.

2. Methods and materials

CALUX-analysis

CALUX analysis was performed as described previously [20]. In short, 5 gram of feed or bakery waste was suspended in 15 ml methanol/water 85/15 (v/v) and extracted with hexane/diethyl ether 97/3 (v/v). The extract was subsequently cleaned over a 33% acid silica column (10 g). The eluate was dried to a small volume, mixed with 40 µl DMSO and further dried to remove the organic solvent. The DMSO was mixed with 2 ml culture medium, which was then added to the cells (0.25 ml to a well containing already 0.25 ml medium). After a 24 h incubation period cells were lysed and the luciferase measured using an Ascent luminometer (Thermo Labsystems). The response was compared to that of a set of chicken feed samples containing 0.02, 0.34, 0.58, 0.76, 1.84, and 3.95 ng TEQ/kg of a mixture of dioxins and dioxin-like PCBs, with a relative contribution of dioxins, no-PCBs and mo-PCBs to the total TEQ of 50, 30 and 20%. Samples showing a higher response than the sample of 0.6 ng TEQ/kg were termed suspected and analysed by GC/MS.

Fat from calves (0.5 g) was mixed with hexane/diethyl ether and directly applied to the acid silica columns. In the case of pigs, 2 gram fat was mixed with 5 ml hydro sulphuric acid, incubated overnight at room temperature and subsequently extracted with hexane/diethyl ether. This extract was applied on the acid silica columns. Eventually the extract was evaporated with 20 µl DMSO as keeper, which was them mixed with 1 ml medium. A natural contaminated milk fat sample containing 0.5 ng TEQ/kg and the same fat enriched to levels of 1, 2, 3 and 6 ng TEQ/kg were included in each series, as well as a carbon cleaned fat sample. Samples were declared suspected if the response was higher than the sample containing 0.5 (pig fat) or 2 (calf fat) ng TEQ/kg.

HRGC/HRMS analysis

HRGC/HRMS analysis was performed as described previously by Tuinstra et al.[22], with the exception that 3 gram fat or 50 gram of feed/bakery waste was used. In short, samples were fortified with 13C-labelled standards and subsequently purified on a gel-permeation, an aluminium oxide and an activated carbon column. The two fractions eluting from the last column were analysed for respectively the dioxins and non-ortho PCBs, and the mono-ortho PCBs.

3. Results and discussion

Animal feed and bakery waste

A total of 339 samples were screened within a period of 3 weeks, being 168 feed or bakery waste samples and 171 animal fat samples. Bakery waste samples were traced effectively and all samples showed an increased response in the CALUX assay. The highest level measured by GC/MS was 11.5 ng TEQ/kg, the lowest level 1.0 ng TEQ/kg. Figure 1 shows the TEQ based congener patterns of two samples of bakery waste containing 8.1 and 6.4 ng TEQ/kg, as compared to the sample analysed by the Fresenius Institute. It is evident that the profiles are identical with 2,3,4,7,8-PeCDF (TEF 0.5) being the most important congener, followed by 1,2,3,7,8-PeCDD (TEF 1). In terms of absolute amounts, TCDF, HpCDD and OCDD showed similar levels, but don't contribute to the total TEQ level. The profile was also compared to data presented by Wunderli et al. [23] on congener profiles obtained from the incineration of waste wood. As shown in Figure 1, patterns were very similar, thus confirming the source of the dioxin contamination.

A number of feed samples were found suspected in the CALUX-assay, but contrary to the bakery waste samples, none of these samples exceeded the current limit of 0.75 ng TEQ/kg as determined by GC/MS. This may be explained by the fact that the feeds contained only 10% or less of the contaminated material. Nevertheless, suspected samples always showed levels above background levels in the Netherlands and several exceeded the EU action limit of 0.5 ng TEQ/kg. Table 1 shows the comparison of the results obtained for feed and bakery waste samples with CALUX

and GC/MS, based on this action limit. No false-negative results were obtained and 16 false-positives, in all cases animal feed. As shown in Figure 2, results obtained with CALUX tended to overestimate the dioxin levels. This figure shows a comparison between the GC/MS determined level and the response obtained in the CALUX-assay (expressed in RLU's) for the different samples. The horizontal lines show the response obtained with the different chicken feed samples. In this particular case several of the feed samples showed e.g. a higher response than the control feed containing 1.8 ng TEQ/kg, whereas the GC/MS determined level was below the limit of 0.75 ng TEQ/kg. Similar has been observed in other cases of feed ingredients dried on open fires or contaminated by smoke from accidental fires. A possible explanation might be the presence of other Ah-receptor agonists in the feed. Analysis by GC-TOF confirmed the presence of many other compounds in the dried bakery waste, among them polycyclic aromatic hydrocarbons (PAHs) and methylated PAHs. Whether the compounds are responsible for this effect remains to be determined.

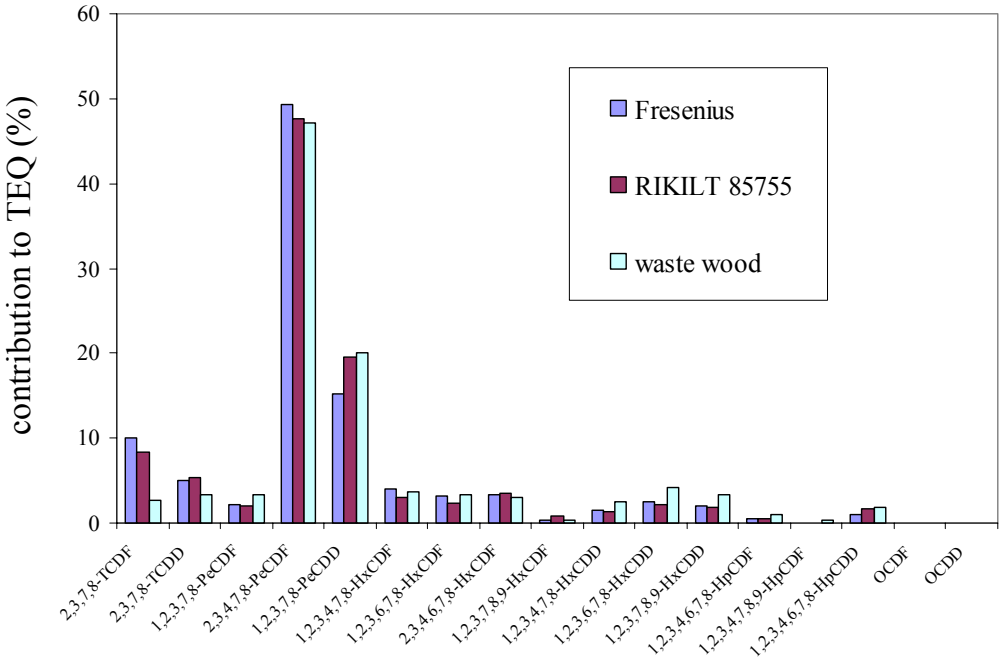

Figure 1. Congener-pattern of dioxins analysed in bakery waste, as analysed by the Fresenius institute in Bayreuth and RIKILT, and compared to a congener-pattern obtained after combustion of waste wood [23]. Levels of each congener are expressed as the relative contribution to the TEQ level.

Animal fat

A total of 171 animal fat samples were screened with CALUX including 10 calf fat samples. The latter samples, derived from animals fed with suspected feed showed no response above the action limit. GC/MS analysis of these samples showed dioxin levels below 0.5 ng TEQ/kg fat. Based on these results, no further calf samples were examined and all investigations focussed on pig fat. For the use of the CALUX-assay this meant that samples had to be examined for exceeding the very low limit of 0.6 ng TEQ/kg, which is the current action limit in the EU. Using the newly developed clean-up procedure, 14 samples out of 171 were declared suspected and 12 of these samples were confirmed by GC/MS (Table 2). Two results were false positive. Furthermore, one negative sample was shown to contain 0.7 ng TEQ/kg, thus representing a false-negative sample. Most positive samples slightly exceeded the limit of 1 ng TEQ/kg, with one sample showing a dioxin level of 2.2 ng TEQ/kg. Figure 3 compares the congener pattern of this sample and that of contaminated bakery waste, thus confirming the source of the contamination.

Table 1. Comparison of the results obtained with CALUX and GC/MS for bakery waste and feed samples.

	GC/MS	
CALUX	Negative	Positive
Negative	33	0
Suspected	16	24

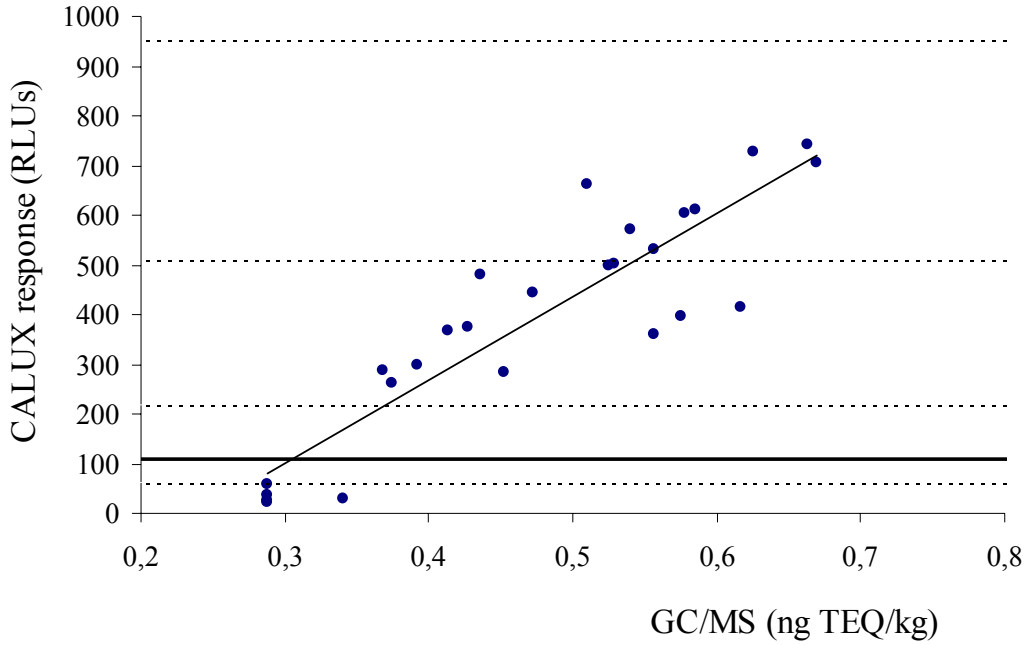

Figure 2. A comparison between the GC/MS determined dioxin level (upper bound levels) and the response in the CALUX (expressed in RLUs). The response obtained with the 5 different chicken feed control samples is included as horizontal lines, going from 0.34, 0.58 (bold line), 0.76, 1.84, 3.95 ng TEQ/kg.

Table 2. Comparison of the results obtained with CALUX and GC/MS for animal fat samples.

	GC/MS	
CALUX	Negative	Positive
Negative	60	1
Suspected	2	12

4. Conclusions

The use of contaminated bakery waste resulted in elevated levels of dioxins in animal feed, but no levels above the limit of 0.75 ng TEQ/kg. The use of contaminated feed resulted in slightly elevated levels in pigs but did not endanger human health. The CALUX bioassay was shown to perform very well, even at these very low limits. Results obtained with CALUX indicate the presence of other unknown Ah-receptor agonists in dried bakery waste.

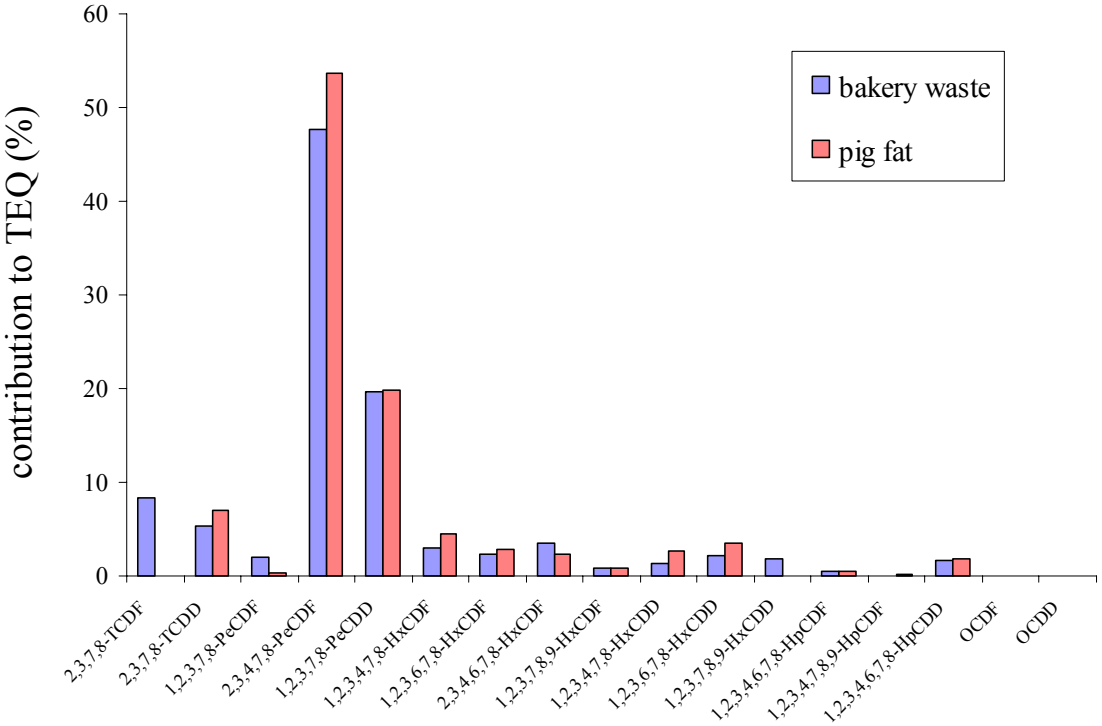

Figure 3. Congener -pattern of dioxins analysed in bakery waste and in pig fat. Levels of each congener are expressed as the relative contribution to the TEQ level.

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Validation of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein, for the screening of estrogenic activity in calf urine

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Abstract

Previously we described the construction and properties of a rapid yeast bioassay stably expressing human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) 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 calf urine. This validation was performed according to EC Decision 2002/657, which prescribes the determination of the detection capability (CCβ), the specificity/selectivity and the stability/ruggedness/applicability. To determine these performance characteristics, twenty blank urine samples of 19 week old calves were collected and spiked with 17β-estradiol (E2β) at 1 ng ml-1, diethylstilbestrol (DES) at 1 ng ml-1, 17α-ethynylestradiol (EE2) at 1 ng ml-1, α-zearalanol at 30 and 50 ng ml-1 or mestranol at 10 ng ml-1. Following enzymatic deconjugation and solid phase extraction, 100 µl equivalents of these blank and spiked urine samples were screened for estrogenic activity in a 96 well plate using the yeast estrogen bioassay. All of these low estrogen spiked urine samples could be distinguished from the blank samples as all spiked samples gave a signal above the determined decision limit CCα and the mean responses of the spiked samples were higher than the determined detection capability CCβ. As this CCβ criterion is met, these spiked samples have a lower than 5% probability to be classified as a false negative. The specificity of the method was determined with blank urine samples spiked with a high dose of testosterone or progesterone (1000 ng ml-1). No response to these substances was detected in the yeast estrogen bioassay. There was also no interference of a high dose of testosterone or progesterone on the response of a low dose of the estrogens. Stability of urine samples was checked with spiked urine samples that were kept frozen for up to 90 days, showing that urine samples could be stored at -20 °C for up to 60 days without changing the screening result of the assay. This method has been in routine use at RIKILT for more than one year.

1 Introduction

The use of growth promoters for fattening purposes in cattle has been banned in the European Union since 1988 [1]. Interestingly, this ban prohibits all substances having hormonal action, rather than providing a list of forbidden hormones. In contrast, residue analysis, while aiming at consumer protection, fair trade and enforcement of the ban, is still carried out on specific target compounds [2]. Despite the limited number of positives found in controls, findings and analysis of illegal preparations show that steroids, natural hormones and β-agonists are still being used [3, 4]. The analytical methods for residues of steroids and β-agonists as used in the control programs are not able to detect very new or outdated compounds and might be one of the possible explanations for the limited positives found so far. The screening and confirmatory gas chromatography/mass spectrometry (GC/MS) and even the multi-residue liquid chromatography tandem mass spectrometry (LC/MS/MS) methods are limited to a short list of a priori known hormone residues [5, 6, 7, 8]. The multi-analyte screening ability of radio- and enzyme immuno assays is dependent on the limited degree of cross-reactivity of the antibody used [9, 10]. Alternatively, receptor based assays can be used to detect all compounds having affinity for a given receptor [11]. 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 [12]. In contrast to competitive ligand binding receptor assays, that cannot distinguish a receptor agonist from an antagonist, transcription activation assays can be used to identify antagonists as well, by giving them in combination with a near maximally effective dose of a known agonist. Although transcription activation assays based on human cell lines are more sensitive than yeast based assays and may be able to identify compounds that require human metabolism for activation into their active state [13, 14], yeast based assays have several advantages. These include robustness, low costs, lack of known endogenous receptors and the use of media that are devoid of steroids [15, 16, 17]. Yeast estrogen bioassays have been used for the rapid determination of estrogenic activity in environmental samples [17,18]. Most of these assays use an extra-chromosomal reporter construct with β-galactosidase as a substrate based reporter protein. Recently we developed a yeast estrogen bioassay, stably expressing human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens. The only handling required to perform this assay is the addition of the yeast suspension to a sample extract, following fluorescence measurement after 4 or 24 h. This assay is completely performed in a 96 well plate and fluorescence is measured directly in intact yeast cells in a plate reader. This yeast estrogen assay is relatively simple and sensitive, as shown by an EC50 value for E2β of 0.5 nM [19]. Furthermore, to validate the assay, a large number of

chemically different compounds with known estrogenic properties were previously tested. All these estrogenic compounds caused a dose-related increase in the production of green fluorescent protein, while the gestagens progesterone and medroxyprogesterone-acetate showed no response and the androgen testosterone only showed a very weak response [20]. In the present study the applicability of this yeast estrogen assay is demonstrated by screening estrogenic activity in calf urine samples. As the Commission Decision of 12 August 2002 of the EC prescribes in EC Decision 2002/657 [21] that validation of a qualitative screening method must include verification of the detection capability (CCβ), specificity and stability, these performance characteristics are determined in the present study for the yeast estrogen assay for the screening of estrogenic activity in calf urine.

2. Experimental

2.1 Chemicals

Water was purified using a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). The β-glucuronidase/arylsulfatase (from suc Helix Pomatia), ammonium sulphate, dimethyl sulfoxide, sodium acetate and sodium carbonate were obtained from Merck (Darmstadt, Germany). The compounds 17β-estradiol (E2β), 17α-ethynylestradiol (EE2), α-zearalanol, mestranol, testosterone, progesterone and L-leucine were purchased from Sigma, diethylstilbestrol (DES) from ICN, Isolute NH2 extraction columns (100 mg) from IST (Hengoed, U.K.) and Bond Elut C18 solid phase extraction columns (500 mg) from Varian (Harbor City, CA, USA). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Difco (Detroit, MI, USA). The minimal medium with L-leucine medium (MM/L) consisted of yeast nitrogen base without amino acids or ammonium sulphate (1.7 g per l), dextrose (20 g per l) and ammonium sulphate (5 g per l) and was supplemented with L-leucine (60 mg per l).

2.2 Sample treatment

The extraction procedure described below is commonly applied to the isolation of steroids from urine samples [22, 23]. Stock solutions of E2β, DES, EE2, α-zearalanol, mestranol, testosterone and progesterone were prepared in DMSO (respectively 0.1; 0.1; 0.1; 5 and 1 μg ml-1). Aliquots of 2 ml of blank calf urine and spiked calf urine samples, E2β, DES and EE2 at 1 ng per ml, α-zearalanol at 30 or 50 ng per ml and mestranol at 10 ng per ml, were adjusted to pH 4.8 and 20 μl β-glucuronidase/arylsulfatase (3 U ml-1) was added. Enzymatic deconjugation was carried out overnight in a water bath at 37 °C. Next, 2 ml of 0.25 M sodium acetate buffer pH 4.8 was added and the hydrolysed sample was subjected to solid phase extraction (SPE) on a C18 column. This column was previously conditioned with 2.5 ml methanol and 2.5 ml sodium acetate buffer. Subsequently, this column was washed with 1.5 ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5 ml sodium acetate buffer pH 4.8, 3.0 ml water and finally with 2 ml methanol/water (50/50 v/v). The column was air-dried and eluted with 4 ml acetonitrile. The eluate was applied to an NH2-column that was previously conditioned with 3.0 ml acetonitrile. The acetonitrile eluate thus obtained was evaporated to 2 ml under a stream of nitrogen gas. A 100 μl part of this extract (equivalent to 100 μl urine) was transferred to a 96 well plate in triplicate and 50 μl water and 2 μl DMSO were added to each well. To get rid of the acetonitrile, the plate was dried overnight in a fume cupboard and was then ready to be screened on estrogenic activities with the yeast estrogen bioassay. In the same way a reagent blank was prepared, using 2 ml of the 0.25 M sodium acetate buffer pH 4.8 instead of urine.

2.3 Yeast estrogen bioassay

The yeast cytosensor expressing the human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) in response to estrogens is developed in-house and was described previously [19, 20]. In short, an agar plate containing the selective MM/L medium was inoculated with the yeast ERα cytosensor from a frozen -80 °C stock (20% glycerol v/v). The plate was incubated at 30 °C for 24-48 h and then stored at 4 °C. The day before running the assay, a single colony of the yeast cytosensor was used to inoculate 10 ml of selective MM/L medium. This culture was grown overnight at 30 °C with vigorous orbital shaking at 225 rpm. At the late log phase, the yeast ERα cytosensor was diluted in MM/L, giving an OD at 604 nm in the range of 0.07-0.13. For exposure in 96 well plates, aliquots of 200 μl of this diluted yeast culture were pipetted into each well, already containing the extracts of the urine samples (see section 2.2). A 17β-estradiol dose-response curve was included in each exposure experiment. Aliquots of 200 μl of the diluted yeast culture were pipetted

into each well of a 96 well plate and exposure to different doses of 17β-estradiol was performed through the addition of 2 μl of 17β-estradiol stock solutions in DMSO. Each urine sample extract and each 17β-estradiol stock was assayed in triplicate. Exposure was performed for 0 h and 24 h. Fluorescence at these time intervals was measured directly in a CytoFluor Multi-Well Plate Reader (Series 4000, PerSeptive Biosystems) using excitation at 485 nm and measuring emission at 530 nm. The densities of the yeast culture at these time intervals were also determined by measuring the OD at 630 nm. This was done to check whether a urine sample was toxic for yeast.

2.4 Assay validation and data analysis

Detection capability CCβ

Extracts of 20 blank calf urine and 20 spiked calf urine samples (E2β, DES and EE2 at 1 ng ml-1, α-zearalanol at 30 or 50 ng ml-1 and mestranol at 10 ng ml-1) were analysed in the bioassay in order to determine the decision limit CCα and detection capability CCβ of the yeast estrogen bioassay. An extract of a reagent blank was made as well and was also analysed in the bioassay. Fluorescence signals of the 20 blank urine samples and the 20 spiked urine samples obtained after 24 h of exposure were corrected for the signals obtained at 0 h (t24-t0) and were also corrected for the signal (t24-t0) obtained with a reagent blank. The extracts of the blank urines and their corresponding spikes were prepared on three different days and were analysed in the yeast estrogen bioassay in three separate exposures. These three sample treatments and exposures were performed within a time period of 10 days. In another experiment, extracts of 20 blank urines and spikes of 50 ng zearalanol per ml urine were prepared in one day and were analysed in the yeast estrogen bioassay in one exposure. In the context of EC Decision 2002/657 [21] we define the mean signal of 20 blank calf urine samples plus 2.33 times the corresponding standard deviation as the decision limit CCα (α=1%) and define the detection capability CCβ as the decision limit CCα plus 1.64 times the standard deviation of the signal of the spiked calf urine sample (β=5%).

Specificity

To determine the specificity of the yeast estrogen bioassay, three blank calf urine samples were spiked with a high dose of testosterone or progesterone (1000 ng ml-1) and extracts were analysed in the bioassay. To check for interference, these three blank calf urine samples were spiked with the high dose of either testosterone or progesterone in combination with a low dose of estrogens (E2β, DES and EE2 at 1 ng ml-1, α-zearalanol at 50 ng ml-1 and mestranol at 10 ng ml-1).

Stability

For the determination of the stability of the urine samples, a pool of blank calf urine was made. Aliquots of 2 ml of this blank calf urine pool and spiked blanks (E2β, DES and EE2 at 1 ng ml-1, α-zearalanol at 50 ng ml-1 and mestranol at 10 ng ml-1) were kept frozen at -20 °C. At certain times, samples were thawed and extracts were made that were analysed in the bioassay.

3. Results and Discussion

The performance characteristics detection capability CCβ, specificity and stability of the yeast estrogen bioassay for the screening of estrogenic activity in calf urine were determined in order to validate the bioassay and to test its possible use to screen low levels of estrogens in calf urine. There is no permitted limit for estrogens, but in routine use 1 ng of 17β-estradiol per ml urine is used as an action level. Urine samples were therefore spiked at a level of 1 ng ml-1 for E2β, DES and EE2, 30 or 50 ng ml-1 for α-zearalanol and 10 ng ml-1 for mestranol. Theoretically, that is if there is no recovery loss during the sample treatment, the equivalent of 100 μl urine of the 1 ng ml-1 17β-estradiol spike that is added to a well in a final well volume of about 250 μl results in a final concentration of 1.5 nM 17β-estradiol. We have shown previously [19, 20] that in the yeast bioassay the concentration where half-maximal activation is reached (EC50), is about 0.7 nM for 17β-estradiol and so theoretically the 1 ng ml-1 spike of 17β-estradiol should easily be detected. In [20] we showed that the relative estrogenic potency (REP) of a compound, defined as the ratio between the EC50 of 17β-estradiol and the EC50 of that compound, is 1, 1.2, 0.033 and 0.11 for DES, EE2, α-zearalanol and mestranol respectively.

This means that both DES and EE2 are about as potent as E2β and that α-zearalanol and mestranol are respectively 30 and 10 times less potent than E2β. Therefore, both DES and EE2 were spiked at the same level as E2β (1 ng ml-1) and α-zearalanol and mestranol were spiked at respectively 30 and 10 times higher levels.

3.1 Detection capability (CCβ)

Table 1 shows the results of the 20 blank and the 20 spiked urine samples. The signals are the responses obtained after 24 h of exposure that are corrected for the responses obtained at 0 h (t24-t0) and that are also corrected for the response (t24-t0) obtained with a reagent blank. All these responses are the mean of a triplicate and in general the %CV of these triplicates is less than 5% (data not shown). After 24 h of exposure there were no differences in the OD at 630 nm, meaning that no toxic effects on the yeast could be observed (data not shown). The blank urine samples #4, #5, #17 and #19 have corrected responses that have a negative value (see Table 1). Negative values are not due to low responses of these blank urines, but are rather caused by relatively little higher responses of the corresponding reagent blanks (see figure 3). The mean response value of the 20 blank urines XB is 59 with a corresponding standard deviation SB of 99. As a result the calculated decision limit CCα for the corrected fluorescence response is 290. This means that sample #7, which gives a response of 364, is either a false positive or not a real good blank. As the rate of false non-compliant results is 1% (α=1%), it is not expected to find a false positive result, but it is statistically possible to have one false positive in a single series of 20 samples. However, when this sample #7 is left out in the calculations, the XB and corresponding SB become 43 and 69 respectively. The CCα value for the corrected fluorescence response becomes 204 and as a result sample #1 now turns into another false positive or is not a real good blank also. When samples #1 and #7 are both left out in the calculations, the XB and corresponding SB become 32 and 52 respectively. For the decision limit this resulted in a CCα value for the corrected fluorescence response of 153. Now all the 18 remaining blank samples have a response that is lower than this CCα value of 153. As samples with signals above this CCα value are classified as suspect, the blank urine samples #1 and #7, which are left out in the latter calculation, would be classified as suspect. To determine whether samples #1 and #7 are false positives or not good representatives of blank samples, both of these “suspect blank urines” and the blank urines #4, #8, #9 and #20 were analysed with GC/MS for 17β-estradiol, 17α-estradiol and estrone. All analyses showed contents well below the 1 ng ml-1 level, but the urines #1 and #7 showed elevated levels of 17α-estradiol, respectively 1.9 and 1.5 ng ml-1 (data not shown) and thus both of these urines are not good representatives of blank urine samples. The results of urine samples #1 and #7 were therefore not included in the calculations. This resulted in a CCα value of 153. As can been seen in Table 1, all E2β, DES, EE2 and mestranol spikes give a response that is higher than this CCα and are thus classified as suspect samples. Only the 30 ng ml-1 α-zearalanol spike in urine samples #4, #5, #9, #12, #14 and #17 give a response that is lower than the CCα and thus would be classified as negative. These negatives are then false compliant results.

Table 2 shows the calculated decision limit CCα, the mean response value of the spike XS, the standard deviation of the spike SS, the calculated detection capability CCβ, a column that shows whether the CCβ criterion was met, two columns that show the calculated contents in ng 17β-estradiol equivalents per ml urine corresponding to respectively the determined CCβ response value and the mean fluorescence response value of the spike XS, a column that shows the relative estrogenic potency (REP) of the compound and the latter column shows the recovery. In order to have a lower than 5% probability on false negative results, the mean response value of the spike Xs has to be higher than the corresponding CCβ. Table 2 shows that the E2β, DES, EE2 and mestranol spikes meet this CCβ criterion (XS > CCβ) and that the 30 ng ml-1 α-zearalanol spike does not fulfil the CCβ criterion. It is thus not unexpected that all individual E2β, DES, EE2 and mestranol spikes give a response that is higher than the CCα value of 153 and are thus classified as suspect samples and that some individual 30 ng ml-1 α-zearalanol spikes give a response that is lower than the CCα and thus would be classified as negative, which are false compliant results. This compound does not fulfil the CCβ criterion at a level of 30 ng ml-1, but in a second attempt 20 blank urine samples were spiked with 50 ng ml-1. Urine samples #1 and #7 were left out, but in order to have 20 blank samples, urine samples #2 and #5 were spiked and analysed in twofold. The data in Table 1 and 2 show that this 50 ng ml-1 α-zearalanol spike fulfils the CCβ criterion and that all 50 ng ml-1 α-zearalanol spikes give a response that is higher than the corresponding CCα value of 117. Thus all individual 50 ng ml-1 α-zearalanol spikes are classified as suspect samples.

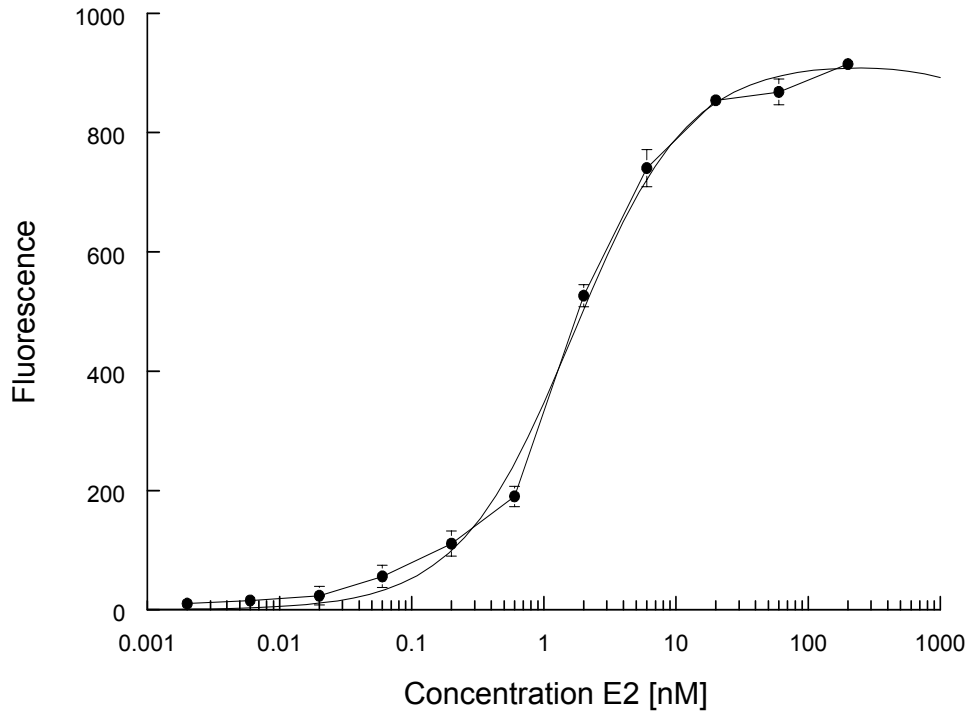

Figure 1. Response of the yeast estrogen cytosensor after a 24 h exposure to 17β-estradiol. Exposure was started by adding 2 μl of a 17β-estradiol stock solution in DMSO to 200 μl of a yeast culture. Fluorescence was determined after 0 and 24 hours as described in Experimental (see section 2.3 Yeast 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.

Although the validation was performed as a qualitative screening method for the determination of estrogenic activity in calf urine, the responses of the urines can be converted to concentrations using a 17β-estradiol standard dose-response curve that is also corrected for a reagent blank (see Figure 1). When the mean response value of the 18 blank urines (see Table 1 for mean response value of 32) is calculated from this standard curve, this quantitative approach results in a concentration of 0.06 nM 17β-estradiol equivalents in the well, which corresponds to 0.04 ng E2β equivalents per ml of blank urine. When the responses of the "suspect blank urines" #1 and #7 are calculated from the 17β-estradiol standard curve, they contained 0.39 and 0.74 ng E2β equivalents per ml respectively and the CCα of 153 corresponds to 0.22 ng per ml. In the same way the mean response values of the spiked samples can be converted to concentrations and contents of E2β equivalents (Table 2). The 17β-estradiol, DES and EE2 spikes are made at a level of 1 ng per ml and as the mean response of the 17β-estradiol spikes corresponds to 0.79 ng E2β equivalents per ml, this means that the recovery of the method for 17β-estradiol is about 79%. For DES, which has a REP of 1, a content of 0.69 ng ml-1 was calculated and thus the calculated recovery for this compound was 69%. For EE2, which has a REP of 1.2, 100% recovery would result in 1.2 ng E2β equivalents per ml and so the calculated content of 0.92 ng E2β equivalents per ml means that the recovery for this compound was about 77%. For α-zearalanol, which has a REP of 0.033 and was spiked at a level of 50 ng per ml, 100% recovery would result in 1.65 ng E2β equivalents per ml and thus the recovery of the method for this compound is about 39%. Mestranol, which has a REP of 0.11, was spiked at a level of 10 ng per ml and so 100% recovery would result in 1.1 ng E2β equivalents per ml. Thus the calculated content of 1.10 E2β equivalents per ml means that the recovery for this compound was about 100%. Although this quantitative approach is beyond the scope of this paper and is not needed for the validation of a

qualitative screening method, it proves that the recovery of the sample treatment is about 80% for 17β-estradiol, diethylstilbestrol, 17α-ethynylestradiol and mestranol and thus the recovery losses of these compounds are limited. Only the recovery for α-zearalanol is relatively low. However, recovery losses with the applied extraction procedure are mainly determined by the relative polarity of a compound and as α-zearalanol is just as polar as 17β-estradiol [12], high recovery losses for α-zearalanol are not likely. More probable, the determined REP of this compound [20] is a little too high. If the real REP of α-zearalanol is e.g. 0.020 instead of 0.033, the calculated recovery would already be 65%.

3.2 Specificity and interference

The specificity of the yeast estrogen bioassay was determined with three blank calf urine samples that were spiked with a high dose of testosterone or progesterone, both at a level of 1000 ng ml-1. Interference was checked with these three blank urine samples by spiking them with a low dose of E2β, DES, EE2, α-zearalanol and mestranol in combination with the high dose of either testosterone or progesterone. Fluorescence signals at 24 h are corrected for the signals obtained at 0 h and the response of a reagent blank. Results are shown in Figure 2. The data show that neither the androgen testosterone nor the gestagen progesterone give a response in the bioassay, signals below the CCα, demonstrating the specificity of the bioassay. Figure 2 also shows that both of these compounds do not interfere with the screening result of the estrogenic compounds E2β, DES, EE2, α-zearalanol or mestranol, as all signals of the estrogen spiked samples are above the CCα value of 153.

3.3 Stability of estrogens in urine

For the determination of the stability of estrogens in urine, blank urine and spiked urine samples were stored at -20 °C. At certain time intervals, samples were thawed and extracts were made that were analysed in the bioassay. Figure 3 shows the fluorescence response of the blank and spiked sample at 24 h, corrected for the response at 0 h only. This figure also shows the fluorescence response of the reagent blank and a 600 pM 17β-estradiol reference standard (final concentration in the well) (both corrected for the response at 0 h). Figure 3 demonstrates that the urine samples can be stored at -20 °C for up to 60 days, without disrupting the screening result. Only the 1 ng ml-1 DES spike that was stored at - 20 °C for 90 days gave a corrected response that was below that of the CCα value of 153 (corrected for the corresponding reagent blank that gave a response of 219). Figure 3 also demonstrates that triplicates of urine samples in general give CV < 5% and that a blank urine can give a response that is a little lower than that of the corresponding reagent blank. As a result, signals of blank urine samples that are corrected for the response obtained with a reagent blank sometimes have a negative value. More important however, this figure demonstrates that the responses of the blank urine, spiked urine samples, 600 pM 17β-estradiol reference standard and the responses of the corresponding reagent blanks show little variation in time. This means that the method is not only robust, but, as variability is low, is also reproducible.

4. Conclusions

The data presented and the determined performance characteristics prove that the yeast estrogen bioassay can detect low levels of 17β-estradiol (1 ng ml-1), diethylstilbestrol (1 ng ml-1l), 17α-ethynylestradiol (1 ng ml-1), α-zearalanol (50 ng ml-1l) and mestranol (10 ng ml-1) in calf urine. The signals of all of these spiked urine samples were higher than the determined CCα and the mean responses of these spiked samples were higher than the determined CCβ and thus there is less than 5% probability on a false compliant decision when calf urine samples contain equal or higher amounts of these estrogens, that is when calf urine samples contain 1 ng 17β-estradiol equivalents or more per ml. High levels of the androgen 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. This demonstrates that the bioassay is specific for estrogenic substances. The fact that the two blank urines that gave a relative high response in the yeast estrogen bioassay, and would as a screening result be classified as suspect samples, contained elevated levels of 17α-estradiol, further demonstrated the specificity and screening capacity of this bioassay. That urine samples could be stored at - 20 °C for up to 60 days without influencing the screening result showed that the procedure is robust. The stability study also demonstrated that the procedure is reproducible. As all these performance characteristics met the criteria that were put forward in EC Decision 2002/657 [21] for

Table 1. Mean (X) fluorescence response in the yeast estrogen bioassay of 20 blank and 20 spiked urine samples and the determination of the decision limit CCα.

				Day 1								Day 2								Day 3			
Urine sample #	**Mean (X)[a]**	**SD (S)[a]**	**CCα[a]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Blank urine	32	52	153	239	45	48	-29	-32	33	364	64	45	76	23	54	39	18	49	79	-53	83	-85	121
E2beta 1 ng ml^{-1}	379	66		370	489	456	334	338	410	429	481	342	381	373	278	334	317	285	350	469	391	443	346
DES 1 ng ml^{-1}	351	52		341	387	347	340	316	367	446	409	342	341	334	302	301	307	381	332	241	453	374	439
EE2 1 ng ml^{-1}	413	89		438	524	471	338	372	413	534	474	366	364	378	338	340	336	352	358	369	659	536	442
zearalanol 30 ng ml^{-1}	199	85		332	340	195	114	133	298	456	388	150	214	197	147	175	135	208	167	66	186	153	316
mestranol 10 ng ml^{-1}	456	98		417	590	629	375	410	393	654	594	469	363	407	409	395	379	510	413	343	395	486	648
Urine sample #	**Mean (X)[b]**	**SD (S)[b]**	**CCα[b]**	**2**	**2**	**3**	**4**	**5**	**5**	**6**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Blank urine	22	41	117	9	14	10	75	8	32	112	19	-8	26	13	9	-8	-31	-62	11	37	108	43	29
zearalanol 50 ng ml^{-1}	337	47		315	298	425	408	301	265	324	421	364	355	320	349	301	350	384	269	367	304	312	315

[a] Mean, SD and CCα (CCα = Mean of the blank + 2.33*SD of the blank) are determined from 18 urine samples, as the results of urine #1 and #7 were left out the calculations because they are not real good representatives of blank urines (contained more than 1 ng 17α-estradiol per ml). The extracts were prepared on three different days and were analysed in the yeast estrogen bioassay in three separate exposures. These sample treatments and exposures were performed within a time period of 10 days.

[b] Mean, SD and CCα are determined from 20 urine samples, but these are 18 different samples and urine samples #2 and #5 were analysed in twofold. The extracts were prepared in one day and were analysed in the yeast estrogen bioassay in one exposure.

Table 2. Determination of the detection capability CCβ, the calculated contents in 17β-estradiol equivalents per ml urine and the corresponding recovery of the spike.

Component	CCα[a]	Mean[b] X_S	SD[b] S_S	CCß[c]	CCß criterion[d] met (X_S >CCß)	CCß content[e] [ng E2 equivalents ml^{-1}]	Content[f] [ng E2 equivalents ml^{-1}]	REP[g]	Recovery[h] [%]
E2 1 ng ml^{-1}	153	379	66	261	yes	0.44	0.79	1.0	79
DES 1ng ml^{-1}	153	351	52	238	yes	0.39	0.69	1.0	69
EE2 1 ng ml^{-1}	153	413	89	299	yes	0.54	0.92	1.2	77
zearalanol 30 ng ml^{-1}	153	199	85	292	no	0.52	0.31	0.033	31
zearalanol 50 ng ml^{-1}	117	337	47	194	yes	0.30	0.65	0.033	39
mestranol 10 ng ml^{-1}	153	456	98	314	yes	0.59	1.10	0.11	100

[a] Determined CCα (see Table 1).
[b] Mean fluorescence response (X_S) and the corresponding SD (S_S) of the spiked urine sample in the yeast estrogen bioassay (see Table 1).
[c] Determined CCβ (CCβ = CCα + 1.64*SD of the spiked sample).
[d] The CCβ criterion is met if the mean fluorescence response value of the spike is equal to or higher than the corresponding determined CCβ (X_S > CCβ).
[e] The calculated content in ng E2β equivalents per ml urine corresponding to the determined CCβ value.
[f] The calculated content in ng E2β equivalents per ml urine corresponding to the mean fluorescence response value of the spike.
[g] The relative estrogenic potency (REP) of the compound (obtained from [20]).
[h] The recovery. The recovery is calculated by dividing the calculated content[f] by the product of the spiked content [ng ml^{-1}] and the REP and multiply that with 100%.

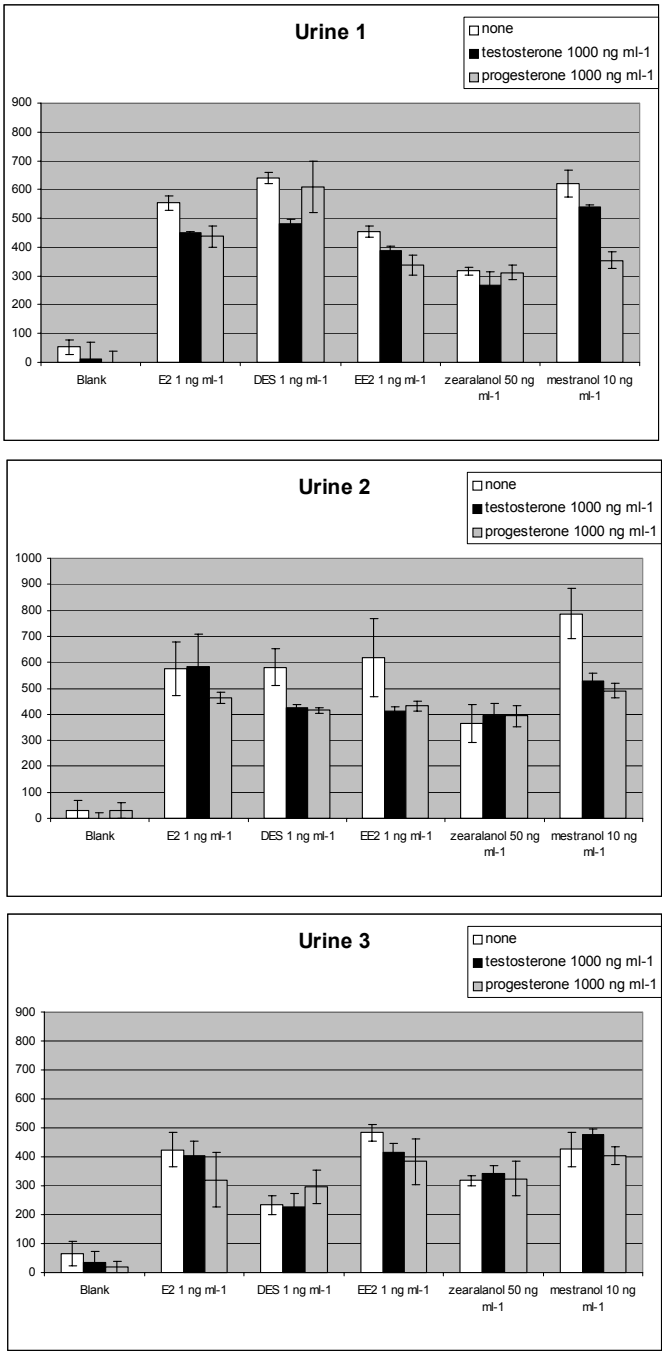

Figure 2. Specificity and interference of the yeast estrogen bioassay checked with urine samples spiked with a high dose of testosterone or progesterone. The specificity of the yeast estrogen bioassay was determined with three blank urine samples that were spiked with a high dose of testosterone or progesterone, both at a level of 1000 ng per ml. Interference was checked with the same three urine samples by spiking them with a low dose of E2β, DES, EE2, α-zearalanol and mestranol in combination with the high dose of either testosterone or progesterone. Extracts were made as described in Experimental (see section 2.2 Sample treatment). Fluorescence signals are the mean of a triplicate with sd and are corrected for the signals obtained at 0 h and a reagent blank.

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 calf urine. This clean-up procedure for the urine 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 bioassay that is suited to be used as a high throughput system for the screening of estrogenic activity in complex calf urine samples in which there are more endocrine active substances than estrogens only. This method is in routine use at RIKILT for more than one year now and showed excellent robustness: urine extracts were never cytotoxic, blank urines were always found compliant and spiked control samples were always screened suspect. However, the method is validated as a qualitative screening method and thus suspect screened samples need to be confirmed. Therefore, we have set up a liquid chromatochraphy (LC) system that fractionates urine extracts into a 96 well fraction collector via effluent splitting. The LC-fractions in the first 96 well plate are used for the detection of estrogenic activity using the yeast estrogen bioassay. The positive well numbers are than correlated with the LC retention time and analyses of the corresponding positive wells in the second 96 well plate with QTOFMS/MS with exact mass measurement. This approach enables us to identify residues of known and unknown estrogens in calf urine [12]. In addition, we have just set up the same procedure for the determination of estrogenic activity in animal feed. Feed samples were extracted with a mixture of 0.25 M sodium acetate buffer pH 4.8 and methanol (50/50 v/v) and extracts were subsequently subjected to the same solid phase extraction procedure as urine samples.

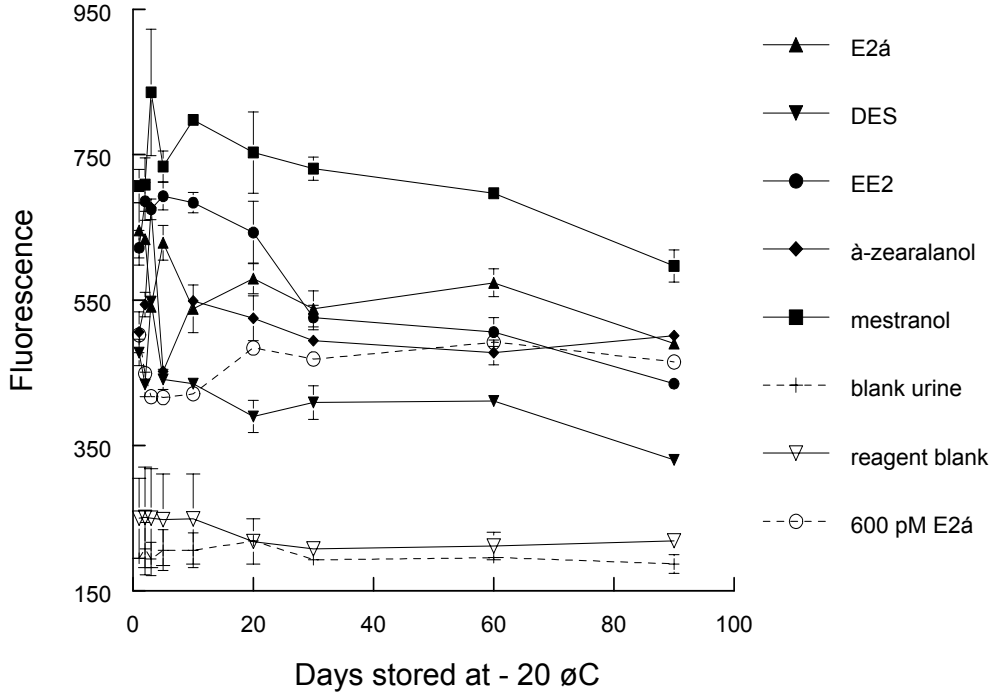

Figure 3. Stability of estrogens in calf urine stored at -20 °C. For the determination of the stability of the estrogens in urine, blank and spiked urine samples were kept frozen at -20 °C. At certain time intervals samples were thawed and extracts were made that were analysed in the bioassay (see Experimental). Also shown is the response of a reagent blank and a 600 pM reference standard of 17β-estradiol. Fluorescence signals are the mean of a triplicate with sd and are only corrected for the signals obtained at 0 h.

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7

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 α (hERα) 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 17β-estradiol (E2β) at 5 ng g-1, 17α-ethynylestradiol (EE2) at 5 ng g-1, diethylstilbestrol (DES) at 10 ng g-1, zearalenone at 1.25 µg g-1 or equol at 200 µg g-1. All of these blank and low estrogen spiked feed samples fulfilled the CCα and CCβ criterions, meaning that all 20 blank feed samples gave a signal below the determined decision limit CCα and were thus classified as compliant and at least 19 out of the 20 spiked samples gave a signal above this CCα (β=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. This is the first successful example of a developed, validated and applied bioassay for the screening of hormonal substances in feed.

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 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 to 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.

Several assays have been developed for this purpose, using both mammalian and yeast cells. In general transcription activation assays based on mammalian or more particular human cell lines have been shown to be more sensitive than yeast based assays, and may be able to identify compounds that require human metabolism for activation into their active state (Legner et al., 1999; Hoogenboom et al., 2001; Sonneveld et al. 2005). However, yeast based assays have several other advantages. These include low costs and easier handling, lack of known endogenous receptors that may compete with the activity under investigation, use of media that are devoid of steroids (Breithofer et al., 1998; Graumann et al., 1999; Witters et al., 2001), and last but not least, yeast cells assays are extremely robust and survive extracts from dirty sample matrices such as urine (Bovee et al., 2005; Nielen et al., submitted). Recently we developed a novel yeast estrogen bioassay (REA), stably expressing human estrogen receptor α (hERα) and expressing yeast enhanced green fluorescent protein (yEGFP) in response to estrogens. The only handling required to perform this assay is the addition of the yeast suspension to a sample extract, followed by fluorescence measurement after 24 h. This assay is completely performed in a 96 well plate and fluorescence is measured directly in intact yeast cells in a plate reader. This assay is relatively simple and sensitive, as shown by an EC50 value for E2β of 0.7 nM (Bovee et al., 2004a). Furthermore, to investigate the specificity of the assay, a large

number of chemically different compounds with known estrogenic, but also other hormonal activities were tested. All these estrogenic compounds caused a dose-related increase in the production of green fluorescent protein, whereas the gestagens progesterone and medroxyprogesterone 17-acetate showed no response and the androgen 17β-testosterone only showed a very weak response (Bovee et al., 2004b). This bioassay has been fully validated for calf urine (Bovee et al., 2005) and recently acquired an ISO17025 accreditation status in The Netherlands for this matrix.

The present study describes the validation of the assay for animal feed. Regulation (EC) No 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, that shall apply from 1 January 2006, prescribes that laboratories involved in the analysis of official samples should work in accordance with internationally approved procedures or criteria-based performance standards and use methods of analysis that have, as far as possible, been validated. Therefore, the yeast estrogen bioassay for the screening of estrogenic activity in animal feed was validated as the Commission Decision of 12 August 2002 of the EC prescribes in EC Decision 2002/657. As this decision prescribes that validation of a qualitative screening method must include verification of the detection capability (CCβ), specificity and stability, these performance characteristics are determined in the present study. 17β-Estradiol (E2β), 17α-ethynylestradiol (EE2), diethylstilbestrol (DES), zearalenone and equol were chosen as model compounds, because E2β, EE2 and DES are priority compounds for both hormone abuse and pharmaceutical waste and because of the differences in their polarity, with equol being the most polar and DES being the most a-polar compound. Furthermore, zearalenone and equol are natural compounds that may occur in animal feed. Zearalenone is an estrogenic mycotoxin and equol is an estrogenic metabolite of the less estrogenic phytoestrogen daidzein. As representatives for feed samples, 7 wet pulp feed samples (pigs), 7 dry grinded feed samples (ruminants, poultry, pigs) and 6 regular milk replacer samples (calves) were used. In this study we also present long-term performance data for the screening of estrogenic activity in animal feed. The applicability of this early warning bioassay system was further demonstrated by screening and identifying the estrogenic activity found in wet pulp feed samples of the MPA-incident and in the routine screening of fish feed samples.

2. Experimental

2.1 Chemicals

Water was purified using a Milli-Q Gradient A10 system (Millipore, Bedford, MA, USA). Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). Ammonium sulphate, dimethyl sulfoxide, sodium acetate and sodium carbonate were obtained from Merck (Darmstadt, Germany). The compounds 17β-estradiol (E2β), 17α-ethynylestradiol (EE2), zearalenone, 17β-testosterone, progesterone and L-leucine were purchased from Sigma,. equol from Apin Chemicals (Abingdon, U.K.), diethylstilbestrol (DES) from ICN. Isolute NH2 extraction columns (100 mg) from IST (Hengoed, U.K.) and Bond Elut C18 solid phase extraction columns (500 mg) from Varian (Harbor City, CA, USA). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Difco (Detroit, MI, USA). The minimal medium with L-leucine medium (MM/L) consisted of yeast nitrogen base without amino acids and without ammonium sulphate (1.7 g per l), dextrose (20 g per l) and ammonium sulphate (5 g per l) and was supplemented with L-leucine (60 mg per l).

2.2 Samples and sample treatment

As representatives for 20 animal feed samples, 7 wet pulp feed samples which are normally used to feed pigs, 7 dry grinded (1 mm) feed samples which are normally used to feed ruminants, poultry and pigs and 6 regular milk replacers which are normally used to feed calves were used. The extraction procedure described below is partly comparable to the procedure that is commonly applied to the isolation of steroids from urine samples (Blankvoort et al., 2003; Nielen et al., 2004). Stock solutions of E2β, EE2, DES, zearalenone, equol, testosterone and progesterone were prepared in DMSO (respectively 0.5; 0.5; 0.5; 100, 10000, 50 and 50 μg ml-1). Feed samples were spiked with E2β, EE2 and DES at 5 and 10 ng per g, zearalenone at 1250 ng per g and equol at 200 µg per g. For extraction, 1 g of blank feed and spiked feed samples were mixed with 4 ml methanol and 4 ml sodium acetate pH 4.8. Samples were incubated for 10 min in an ultrasonic bath and subsequently mixed for 15 min head over head. Samples were centrifuged at 3500 g and 4 ml of the upper liquid phase was brought in a glass tube. Next, the pH was adjusted to 4.8 using 4 N acetic acid and the extract was subjected to solid phase extraction (SPE) on a C18 column. This column was previously conditioned

with 2.5 ml methanol and after that with 2.5 ml sodium acetate pH 4.8. Subsequently, this column was washed with 1.5 ml 10% (w/v) sodium carbonate solution, 3.0 ml water, 1.5 ml sodium acetate pH 4.8, 3.0 ml water and finally with 2 ml methanol/water (50/50 v/v). The column was air-dried and eluted with 4 ml acetonitrile. The eluate was applied to an NH2-column that was previously conditioned with 3.0 ml acetonitrile. The acetonitrile eluate thus obtained was evaporated to 3 ml under a stream of nitrogen gas. A 100 µl or 200 µl aliquot of this extract, equivalent to respectively 0.017 or 0.033 g animal feed, was transferred to a 96 well plate in triplicate and 50 µl of a 4% DMSO solution was added to each well. To remove the acetonitrile, the plate was dried overnight in a fume cupboard and was then ready to be screened on estrogenic activities with the yeast estrogen bioassay. In the same way and in each series a reagent blank was prepared.

2.3 Yeast estrogen bioassay

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

2.4 Assay validation and data analysis

Detection capability CCβ

Extracts of 20 blank animal feed and 20 spiked animal feed samples (E2β, EE2 and DES at 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1) were analysed in the bioassay in order to determine the decision limit CCα and the detection capability CCβ 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 (t24-t0) and were also corrected for the signal (t24-t0) 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 µl aliquots were analysed in the yeast estrogen bioassay in five separate exposures. The 200 µl 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 E2β, EE2 and DES at 5 ng g-1 were prepared in two days and 200 µl 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 CCα (α=1%). Samples with a signal below this CCα are classified as compliant and samples with a signal above this CCα are classified as suspect. The criterion for the decision limit CCα is that all 20 blank feed samples give a signal below the determined decision limit CCα and are thus classified as compliant (α=1%). The criterion for the detection capability CCβ is that at least 19 out of the 20 spiked samples have to give a signal above this CCα and are thus classified as suspect (β=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, were spiked with a high dose of 17β-testosterone or progesterone (1000 ng g-1) and 200 µl extracts were analysed in the bioassay. To check for interference, these three blank feed samples were spiked with the high dose of either 17β-testosterone or progesterone in combination with a low dose of estrogens: E2β, EE2 and DES at 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1.

Stability

For the determination of the stability of the feed samples, aliquots of 1 g of a blank and spiked wet feed sample were stored at -20°C and aliquots of 1 g of a blank and spiked dry feed sample and of a blank and spiked milk replacer were stored at room temperature in the dark. At certain times, 1 g aliquots of these three blank and spiked feed samples: E2β or EE2 at 5 ng g-1, DES at 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1, were taken and extracts were made and 200 µl aliquots were analysed in the bioassay.

3. Results and discussion

The performance characteristics detection capability CCβ, specificity and stability of the yeast estrogen bioassay for the screening of estrogenic activity in animal feed were determined in order to validate the bioassay and to test its possible use to screen low levels of estrogens in animal feed. There is no permitted limit for estrogens, but for the routine screening an action level of 5 ng of 17β-estradiol per gram feed was selected for validation. This is very low, as with illegal use or accidents with pharmaceuticals, much higher contents are expected. Theoretically, assuming no recovery loss during the sample treatment, the 200 µl extract, equivalent to 0.033 g of feed, of the 5 ng g-1 17β-estradiol spike that is added to a well in a final well volume of about 250 µl, results in a final concentration of 2.45 nM 17β-estradiol in the well. We have shown previously (Bovee et al., 2004b) that in the yeast bioassay the concentration where half-maximal activation is reached (EC50), is about 0.7 nM for 17β-estradiol and so theoretically the 5 ng g-1 spike of 17β-estradiol should easily be detected. Bovee et al. 2004b showed that the relative estrogenic potency (REP) of a compound, defined as the ratio between the EC50 of 17β-estradiol and the EC50 of that compound, is 1.2, 1, 0.005 and 0.0005 for EE2, DES, zearalenone and equol respectively. This means that both EE2 and DES are about as potent as E2β and that zearalenone and equol are respectively 200 and 2000 times less potent than E2β. Therefore, both EE2 and DES were spiked at the same level as E2β (5 ng g-1) and zearalenone and equol were spiked at higher levels, respectively 1.25 and 200 µg g-1. With the used clean-up procedure it is expected that the more apolar compounds will have slightly lower recoveries. DES, the most apolar compound tested, was therefore also spiked at the two times higher level of 10 ng g-1.

In the routine screening of animal feed samples, the 200 µl extracts were occasionally (<1% of the samples) cytotoxic. In order to be able to screen these extracts on estrogenic activity, 100 µl instead of 200 µl aliquots of these extracts were analysed in the bioassay. The 100 µl aliquots never showed cytotoxicity. In a second validation experiment, feed samples were spiked with E2β, EE2 and DES at 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1 and 100 µl aliquots of the extracts, equivalent to 0.017 g feed, were analysed in the bioassay.

3.1 Detection capability (CCβ) with 200 µl aliquots of the feed sample extracts

In this experiment feed samples were spiked with E2β and EE2 at 5 ng g-1, DES at 5 and 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1. Table I shows the results of the yeast estrogen bioassay of the 20 blank and the 20 spiked feed samples using 200 µl of the extracts. The signals are the responses obtained after 24 h of exposure that are corrected for the responses obtained at 0 h (t24-t0) and for the response (t24-t0) obtained with a reagent blank. All these responses are the mean of a triplicate and in general the %CV of these triplicates is less than 5% (data not shown). After 24 h of exposure there were no differences in the OD at 630 nm, meaning that no toxic effects on the yeast could be observed (data not shown). Most blank feed samples have corrected responses that have a negative value (see Table I). These negative values are due to the relatively low responses of these blank feed samples when compared to the corresponding reagent blank, for which they are corrected. Also the 17β-estradiol standard curve corrected for the response obtained with a reagent blank starts with low negative values (see Figure 1). Furthermore, these negative values are very low when compared to the signals that were measured. For example, the t0

and t24 measurements of sample #1 were respectively 101 ± 3 (standard deviation) and 307 ± 2, resulting in a t24-t0 signal that was 206 ± 2. The corresponding reagent blank gave a signal of 222 ± 4 and as a result the corrected signal for blank feed #1 is -16. However, the signals of the blank dry feed samples are the lowest and although there is no toxic effect, these extracts probably contain substances that give a little inhibitory effect in the bioassay. The mean response value of the 20 blank feed samples XB is -28 with a corresponding standard deviation SB of 35. The calculated decision limit CCα, being the mean plus 3 times the standard deviation, for the corrected fluorescence response is therefore 77. The use of the assay can be seen as a qualitative ON/OFF method. Samples giving a signal lower than the CCα are compliant: OFF. Samples giving a response higher than the determined CCα are suspect: ON. The results in Table I demonstrate that the blank feed samples fulfil the CCα criterion, meaning that all blank feed samples give a response that is lower than the CCα. All blank samples are thus compliant (method result: OFF). For DES spiked at 5 ng per gram feed, 10 of the 20 spiked samples gave a response that is lower than the determined CCα of 77. This compound does not fulfil the CCβ criterion at the 5 ng g-1 level. However, DES spiked at 10 ng g-1 fulfils the CCβ criterion, as all 20 spiked samples gave a response that is higher than the CCα and are thus suspect (method result: ON). All but two of the E2β, EE2, zearalenone and equol spikes gave a response that is higher than the determined CCα and are thus classified as suspect samples. Only sample #4 for E2β and sample #4 for EE2 showed a response that is lower than the CCα and are thus classified as compliant. These are false compliant results. However, overall the results fulfil the CCβ criterion, meaning that at least 19 out of the 20 spiked samples gave a signal above the CCα and are thus classified as suspect (method result: ON) (β=5%).

In general the spikes in milk replacer gave about the same mean signals (X) as all 20 feed samples, spikes in dry feed gave lower responses and spikes in wet feed showed higher responses. Probably the recovery of the spiked compounds is best from wet feed and recoveries from milk replacer and dry feed are lower. Recovery losses with the applied extraction procedure are mainly determined by the relative polarity of a compound and the interaction with matrix particles. So, the lower recoveries from milk replacer and dry feed could be expected as these matrices contain rather apolar particles, just as the compounds tested, and so interactions from the compounds with matrix particles are probably stronger for milk replacer and dry feed and are the strongest for DES, being the most apolar compound of the compounds tested. These recovery losses and/or the possible inhibitory effect in the bioassay of the dry feed extracts are probably the reason for the lower responses found with especially the DES spikes in dry feed. However, the bioassay method is not affected to such an extent that it does disturb the qualitative compliant/suspect (OFF/ON) screening principle of the method.

3.2 Detection capability (CCβ) with 100 µl aliquots of the feed sample extracts

In this experiment feed samples were spiked with 10 ng g-1 of E2β, EE2 and DES, 1.25 µg g-1 of zearalenone or 200 µg g-1 of equol. Table II shows the results of the yeast estrogen bioassay of the 20 blank and the 20 spiked feed samples using 100 µl of the extracts. All these responses are the mean of a triplicate and in general the %CV of these triplicates was less than 5% (data not shown). Again, and as expected, there were no differences in the OD at 630 nm after 24 h of exposure, meaning that no toxic effects on the yeast were observed (data not shown). Just as for the 200 µl aliquots, 100 µl aliquots of the extracts of the blank feed samples have corrected responses with a negative value (see Table II). The mean response value of the 20 blank feed samples XB was -11 with a corresponding standard deviation (SB) of 16. As a result the calculated decision limit CCα for the corrected fluorescence response was calculated to be 37. The results in Table II demonstrate that the blank feed samples fulfil the CCα criterion, meaning that all blank feed samples gave a response that was lower than the CCα. As the rate of false suspect results is 1% (α=1%), it is not expected to find a false suspect result. As can be seen in Table II, all but two of the E2β, EE2, DES, zearalenone and equol spikes gave a response that was higher than the determined CCα and were thus classified as suspect samples. Only sample #2 for DES and sample #19 for equol gave a response that was lower than the CCα and were thus classified as compliant. These are false compliant results. Sample #2 for DES is a real false compliant, but in sample #19 the equol was possibly not spiked. However, all spikes fulfil the CCβ criterion, meaning that at least 19 out of the 20 spiked samples gave a signal above the CCα and are thus classified as suspect (β=5%).

Although the validation was performed as a qualitative screening method for the determination of estrogenic activity in animal feed and is used as an ON/OFF assay, the responses of the feed extracts can be converted to concentrations using a 17β-estradiol standard dose-response curve that is also corrected for a reagent blank (see Figure 1). When the CCα of 37 is calculated from this

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 CCα using 200 µl aliquots of the extracts for exposure.

				day 1										day 2									
Feed sample #	**Mean (X)[a]**	**SD (S)[a]**	**CCα[a]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
				milk	dry	milk	dry	milk	dry	milk	dry	dry	dry	dry	milk	milk	wet	wet	wet	wet	wet	wet	wet
Blank feed	-28	35	77	-16	9	-9	-101	-28	-43	9	-41	-89	-90	-87	-13	-14	-7	-15	-12	-4	-6	-2	-9
E2β 5 ng $g^{-1(b)}$	342	116		300	221	360	**64**	320	372	452	247	128	203	363	411	468	480	447	438	424	406	361	365
EE2 5 ng $g^{-1(b)}$	314	117		299	155	312	**51**	297	307	466	167	97	237	363	382	357	446	417	398	427	411	328	356
DES 5 ng $g^{-1(b)}$	129	123		87	**20**	**35**	**-72**	**36**	118	259	**52**	**-16**	**70**	**36**	**57**	129	290	255	291	278	310	**59**	292
DES 10 ng $g^{-1©}$	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 $g^{-1©}$	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 $g^{-1©}$	342	77		300	414	470	211	303	299	398	283	233	238	420	269	475	387	387	392	323	395	338	302

[a] Mean, SD and CCα (CCα = 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.

(b) 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.

© The extracts were prepared on six different days (see Table 2) and the 200 µl 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.

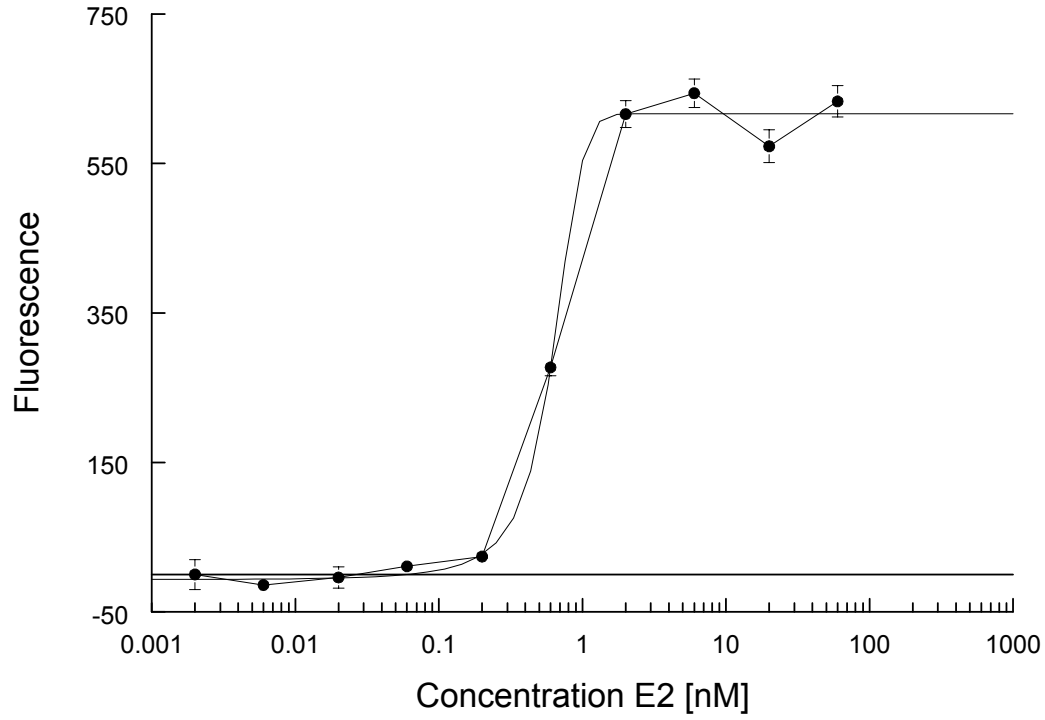

Figure 1. Response of the yeast estrogen cytosensor after a 24 h exposure to 17β-estradiol. Exposure was started by adding 2 μl of a 17β-estradiol stock solution in DMSO to 200 μl of a yeast culture. Fluorescence was determined after 0 and 24 hours as described in Experimental (see section 2.3 Yeast 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.

standard curve this quantitative approach results in a concentration of 0.23 nM 17β-estradiol equivalents in the well. This corresponds to 0.94 ng E2β equivalents per gram of feed. In the same way the mean response values of the spiked samples can be converted to concentrations and contents of E2β equivalents. The 17β-estradiol, EE2 and DES spikes are made at a level of 10 ng per gram and as the mean response of 575 of the 17β-estradiol spikes corresponds to 4.4 ng E2β equivalents per gram, this means that the recovery of the method for 17β-estradiol was 44%. For EE2, which has a relative estrogenic potency (REP) of 1.2, 100% recovery would result in 12 ng E2β equivalents per gram and so the calculated content of 5.1 ng E2β equivalents per gram means that the recovery for this compound was 42%. For DES, which has a REP of 1, a content of 2.3 ng E2β equivalents per gram was calculated and thus the calculated recovery for this compound was 23%. Zearalenone has a REP of 4.6E-3 and was spiked at a level of 1250 ng per gram, thus 100% recovery would result in 5.8 ng E2β equivalents per gram. A content of 2.6 ng E2β equivalents per gram was calculated and thus the recovery of the method for this compound was 45%. The recovery for equol could not be calculated as this compound was spiked with a higher amount, resulting in a mean response value that was equal to the maximum response of the 17β-estradiol standard dose-response curve. Although this quantitative approach is beyond the scope of this paper and is not needed for the validation of a qualitative screening method, it showes that the recovery of the sample treatment is about 45% for 17β-estradiol, 17α-ethynylestradiol and zearalenone. For DES, the most apolar compound tested, the recovery is about 25%.

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 CCα using 100 µl aliquots of the extracts for exposure.

				day 1			day 2		day 3				day 4					day 5				day 6	
Feed sample #	**Mean (X)[a]**	**SD (S)[a]**	**CCα[a]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
				milk	dry	wet	milk	dry	milk	wet	wet	wet	dry	milk	dry	wet	wet	dry	dry	dry	wet	milk	milk
Blank feed	-11	16	37	-11	12	-4	9	-32	-10	-4	-6	-34	-26	4	-31	-5	-6	-36	6	-37	1	-10	5
E2β 10 ng g^{-1}	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^{-1}	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^{-1}	239	164		68	**36**	208	129	80	71	341	292	335	303	418	148	558	421	96	193	118	587	116	263
zearalenone 1.25 µg g^{-1}	312	180		116	92	216	152	125	139	379	292	379	533	734	389	544	358	147	221	351	601	247	226
equol 200 µg g^{-1}	658	303		313	353	632	736	339	466	734	520	742	887	1101	920	1039	938	383	860	842	1011	**-15**	366

[a] Mean, SD and CCα 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 17β-testosterone (A) or progesterone (B).

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

[a] The specificity of the yeast estrogen bioassay was determined with three blank feed samples that were spiked with a high dose of 17β-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 E2β, EE2, DES, zearalenone or equol in combination with the high dose of either 17β-testosterone or progesterone. Exposures were performed with 200 µl aliquots of the extracts. In bold and grey the samples that not fulfil the criterion.

3.3 Specificity and interference

The specificity of the yeast estrogen bioassay was determined with three blank animal feed samples, one wet, one dry and one milk replacer that were spiked with a high dose of 17β-testosterone or progesterone (1000 ng g-1). Interference was checked with these three blank animal feed samples by spiking them with a low dose of E2β, EE2, DES at ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1 in combination with the high dose of either 17β-testosterone or progesterone. Extracts were made and 200 µl aliquots were analysed in the bioassay. Fluorescence signals at 24 h were corrected for the signals obtained at 0 h and the response of a reagent blank. Results are shown in Table III. Strictly seen, the milk replacer spiked with 1000 ng progesterone per gram gave a false suspect result. However, this milk replacer spiked with 1000 ng progesterone per gram gave a signal of 80 that was just above the determined decision limit CCα of 77. When looking at the data in Table III, they show that neither the androgen 17β-testosterone nor the gestagen progesterone gave a response in the bioassay, as the blank samples gave about the same response as the corresponding 17β-testosterone and progesterone spiked samples. Results in Table III also show that both of these compounds do not interfere with the screening result of the estrogenic compounds E2β, DES, EE2, zearalenone or equol, as all signals of the estrogen spiked samples are well above the CCα value of 77. Moreover, responses of the estrogen spiked samples are about the same as spiking them in combination with the high dose of either 17β-testosterone or progesterone. Despite the one false suspect result, the data demonstrate the specificity of the bioassay.

3.4 Stability of estrogens in feed

The stability of the estrogenic compounds in animal feed samples was determined with three animal feed samples, one wet, one dry and one milk replacer that were spiked. Aliquots of 1 g of a blank and spiked wet feed sample were stored at -20°C and aliquots of 1 g of a blank and spiked dry feed sample and of a blank and spiked milk replacer were stored at room temperature in the dark. At certain times, 1 g aliquots of these three blank and spiked feed samples: E2β or EE2 at 5 ng g-1, DES at 10 ng g-1, zearalenone at 1.25 µg g-1 and equol at 200 µg g-1, were taken and extracts were made and 200 µl aliquots were analysed in the bioassay. Fluorescence signals at 24 h were corrected for the signals obtained at 0 h and the response of a reagent blank. Results are shown in Table IV. Only the equol spike in the wet feed that was stored at room temperature in the dark for 55 days gave a corrected response of -15 that was below that of the CCα value of 77. This is a false compliant. The corresponding blank wet feed gave a response of 379 and is a false suspect. However, it is much more likely that there was an interchange of these two samples. The 200 µl extracts of the dry feed at day 0 were toxic to the yeast cells. This could be seen at the OD at 630 nm, while the OD was about 1.0 for the controls, the wet feed and the milk replacer, the OD at 630 nm with 200 µl extracts of this dry feed was below 0.6 (data not shown). Extracts are considered to be toxic if the OD measured at 630 nm is less than 70% of the OD of the control samples. Extracts of day 0 were kept at 4°C and from day 2 on, exposures with the extracts of this dry feed were performed with 100 µl instead of 200 µl. As a consequence, a CCα value of 37 instead of 77 was applied. The 100 µl aliquots of this dry feed sample were analysed and were never cytotoxic. The 200 µl aliquots of this dry feed sample were not always cytotoxic and when not cytotoxic they fulfilled the criteria (data not shown). The data in Table IV demonstrate that the feed samples can be stored at their specific conditions for up to 98 days, without disrupting the screening result, assuming that the blank and equol spike of the wet feed were interchanged at day 55.

3.5 Application of the yeast estrogen bioassay for the screening of estrogenic activity in animal feed

This method is in routine use at RIKILT for more than one year now. In that period, the method was applied more than 40 times to series of about 20 feed samples and showed good robustness: feed extracts were almost never cytotoxic (less than 1% of the samples) and when cytotoxic, the 100 µl method could be used. Reagent blanks were always found compliant and the spiked reagent blanks were always screened suspect. Figure 2 shows a control chart, showing the decision limit CCα and the corrected responses for the reagent blank, blank feed control, spiked reagent blank and the spiked feed control. Only once a blank feed control was found suspect and once a spiked feed control was found compliant. However, the extract of this spiked feed control was coloured. As this control feed was used several times, showing no colour and when spiked was always found suspect, this indicates that only once the clean-up procedure did not work optimally, resulting in a coloured extract that gave a signal just below the determined CCα. The one time that a blank feed control was found suspect, the signal was just above the determined CCα. Just as for the stability study, the data in Figure 2

demonstrate that the method is robust and that the outcome of the assay, compliant/suspect, is reproducible.

The method is validated as a qualitative screening method for estrogenic activity in feed and thus suspect screened samples need to be confirmed. Therefore, we have set up a liquid chromatochraphy (LC) system that fractionates feed extracts using a dual 96 well fraction collector system via effluent splitting. The LC-fractions in the first 96 well plate are used for the detection of estrogenic activity using the yeast estrogen bioassay. The suspect well numbers are then correlated with the LC retention time and results from the analysis of the corresponding "suspect" wells in the second 96 well plate with QTOFMS/MS with exact mass measurement. Figure 3 shows this generic setup for the fractionation and identification of (un)known bioactive substances using LC/bioassay/QTOFMS(/MS) (Nielen et al. 2004; Nielen et al., in press).

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

[a] Exposures were performed with 100 µl aliquots of the extracts, because 200 µl of these extracts were toxic for the yeast cells. The corresponding CCα value is 37 (see Table 1).
[b] Exposures were performed with 200 µl aliquots of the extracts. The corresponding CCα value is 77 (see Table 2). In bold and grey the samples that not fulfil the criterion.

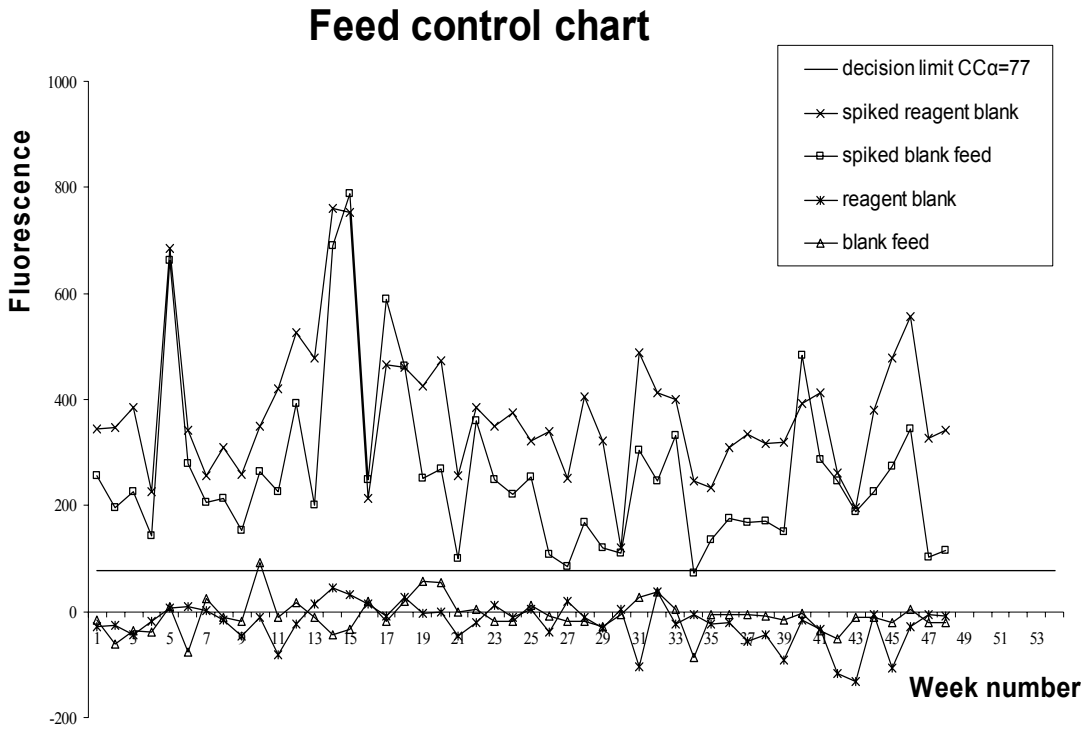

Figure 2. Longterm response (n=48) in the yeast estrogen bioassay of the reagent blank, the reagent blank spiked with 5 ng 17β-estradiol per gram, the blank feed sample and a blank feed sample spiked with 5 ng 17β-estradiol per gram.

During the MPA-incident in the European Union in 2002 there were problems with pregnant pigs in breeding farms (Van Leengoed et al., 2002). To determine the cause of the problems, wet feed samples were send to RIKILT for testing. Part of this testing was to investigate these samples with our estrogen bioassay. The samples were screened suspect in the estrogen bioassay. Knowing that the estrogen bioassay is specific for estrogens, based on the fact that other hormones like 17β-testosterone, progesterone and medroxyprogesterone 17-acetate (MPA) do not give a response in this test (Bovee et al., 2004b), the responsible substances had to be estrogenic. It was decided to fractionate an extract. Figure 4 shows the estrogenicity biogram of a fractionated wet feed and shows that fraction number 24 and 25 are estrogenic. In this system the corresponding retention time of these fractions was equal to that of 17β-estradiol. This was the first indication that hormones could be responsible for the problems in the pregnant pigs and that 17β-estradiol might be involved. This was confirmed by GC/MS analysis. The identified 17β-estradiol turned out to be part of the problem, since early thereafter it became clear that MPA was the main issue in the feed contamination. As hormones used for hormone replacement therapy (HRT) and the female contraceptive are two major pharmaceuticals, both containing large amounts of estrogens, this demonstrates the applicability of the bioassay method as an early warning system for pharmaceutical waste in animal feed. This is even true in cases where the estrogens are part of the contamination, because the test is not influenced by the presence of other hormones, i.e. there is no interference. It also demonstrates that the bioassay can be used to identify a fraction that contains the estrogenic activity.

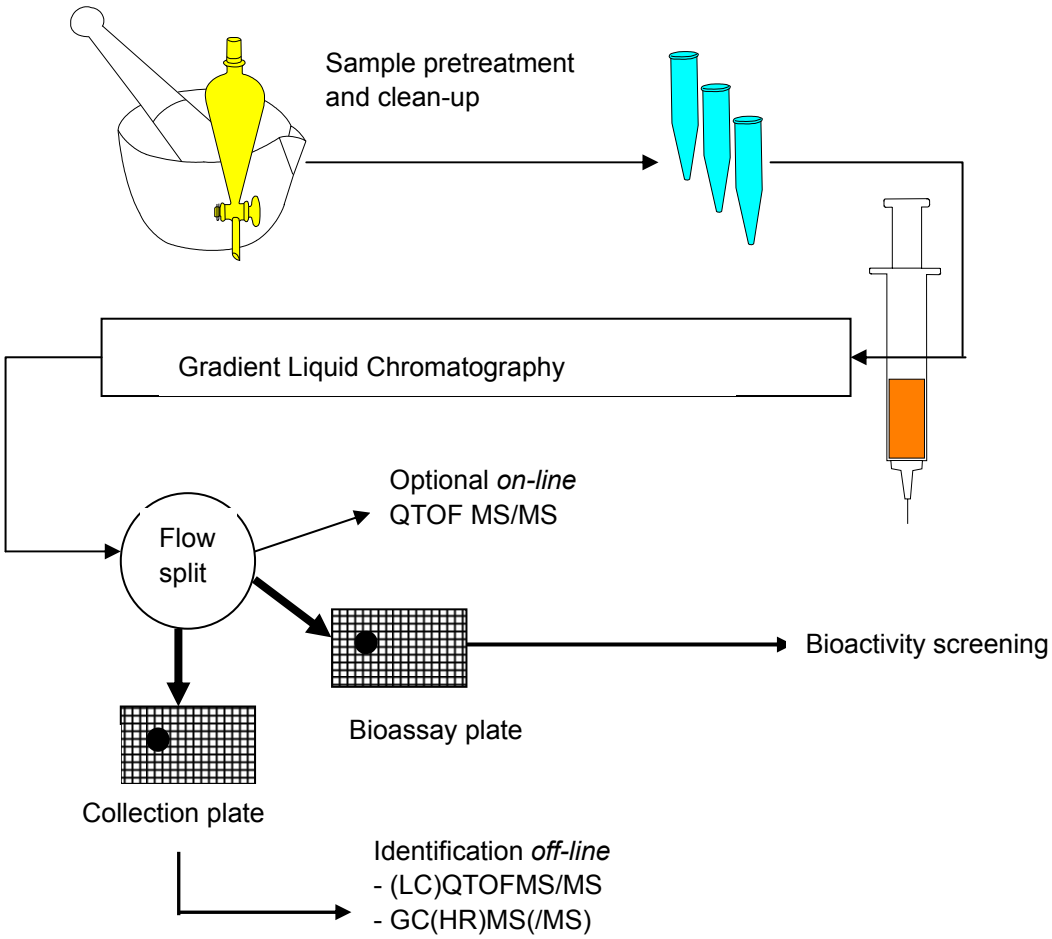

Figure 3. Generic setup for the fractionation and identification of unknown bioactive substances using LC/bioassay/QTOFMS(/MS).

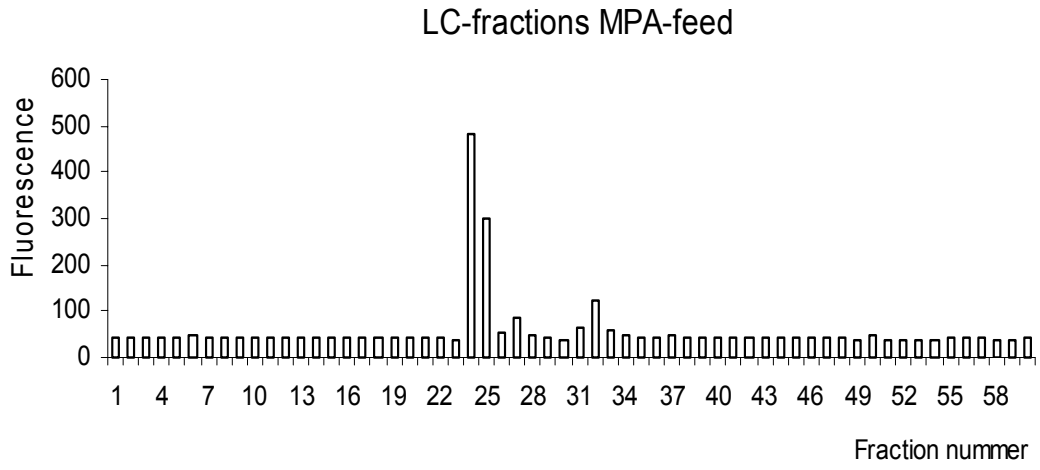

Figure 4. Estrogenicity biogram of a wet feed sample from the MPA-incident that was found suspect in the yeast estrogen bioassay. Extracts were separated on a C18-column and fractions of 0.33 minutes were collected and tested in the bioassay.

Figure 5 shows the estrogenicity biogram of a fractionated fish feed that was found suspect in our routine estrogen bioassay method. Figure 5 shows that fraction number 22 and 23 are estrogenic. In this system (see Figure 3) the corresponding retention time from 7.00 to 7.66 minutes was equal to that of 17β-estradiol. Using the LC/bioassay/MS setup the 17β-estradiol was indeed confirmed by exact mass measurement in both fractions. This demonstrates the applicability of the bioassay method as an early warning system for hormone abuse. It also demonstrates that the bioassay can be used as an inexpensive LC detector to identify a fraction that contains the estrogenic activity. The natural steroids are orally not very active, but the 17-alkylated steroids, like the oral contraceptive 17α-ethynylestradiol, are usually orally active in mammalians and besides as waste, might thus be abused in animal feed. Less well known is the illegal use of hormones in aquaculture. In eel the natural estrogen 17β-estradiol is orally effective. Besides the growth effect, 17β-estradiol affects the sex determination of eels. After 1 year, 70% of eels fed with 60 mg 17β-estradiol/kg in their diet were identified as female, compared to 26% of the control group (Degani and Kushnirov, 1992). In another study these percentages were 88% in the group treated with 17β-estradiol and 6% in the control group. This latter study also showed that after 600 days, males reached a weight of 114,1 ± 4.3 and the females 171 ± 11.7 (g ± SE) (Degani et al., 2003). Thus the abuse of 17β-estradiol for the growth of eel can be very lucrative.

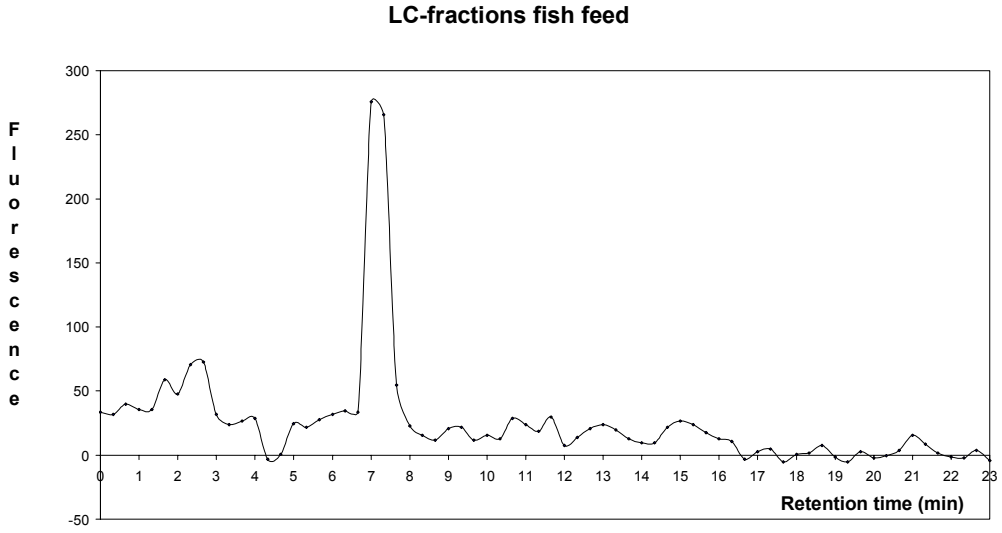

Figure 5. Estrogenicity biogram of a fish feed sample that was suspect in the routine screening. Extracts were separated on a C18-column and fractions of 0.33 minutes were collected and tested in the bioassay.

4. Conclusions

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

High levels of the androgen 17β-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 is needed for the bioassay screening in triplicate while the remaining 2.4 ml can be used for identification and conformation using the LC/bioassay/QTOFMS setup.

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

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8

Summary, concluding remarks and future developments

Summary

This thesis describes the development, validation and routine application of bioassays for detecting the presence of dioxins and estrogenic substances in the food chain. After the introduction to the topic of this thesis in Chapter 1, Chapter 2 describes the validation of the DR-CALUX® bioassay and Chapter 3 and 4 describe the development of the yeast based hERα and hERβ reporter gene assays. Chapter 5 describes the application of the DR-CALUX® during the German bakery waste incident and Chapter 6 and 7 describe the validation and performance of the RIKILT yeast Estrogen bioAssay expressing the hERα (REA) for the screening of estrogenic activity in calf urine and animal feed respectively.

1.1. Optimisation and validation of the DR-CALUX® bioassay

A dedicated cell-line was developed by the Department of Toxicology of Wageningen University in a joined project with the University of California in Davis and the RIKILT-WUR – Institute of Food Safety in Wageningen. In Chapter 2 of this thesis the DR-CALUX® bioassay was tested and optimised for its possible use to determine low levels of dioxins in bovine milk. It was shown that this mammalian cell based test is very sensitive for 2,3,7,8-substituted dioxins and related PCBs, thereby reflecting the relative potencies (TEF) of these compounds as set by the World Health Organisation (WHO). These toxic equivalency factors (WHO-TEFs) express the toxicity of a compound in comparison to the most toxic compound congener 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, TEF=1). The limit of detection was about 50 fg of TCDD. Furthermore, the response obtained with a mixture of dioxins was additive, in accordance with the TEQ-principle. Milk fat was isolated by centrifugation followed by clean-up of the fat with n-pentane, removal of the fat on a 33% H_2SO_4 silica column, and determination of Ah receptor agonist activity with the DR-CALUX® bioassay. An equivalent of 67 mg fat was tested per experimental unit, resulting in a limit of quantification around 1 pg WHO-TEQ per g fat. To investigate the performance of the method, butter fat was cleaned and spiked with a mixture of 17 different 2,3,7,8-substituted PCDD and PCDF congeners at 1, 3, 6, 9, 12 and 15 pg WHO-TEQ per g fat, as confirmed by GC/MS. In this concentration range, the method showed a recovery of TEQs around 67% (58-87%). The reproducibility, determined in three independent series showed a coefficient of variation (CV) varying between 4% and 54%, with the exception of the sample spiked at 1 pg WHO-TEQ/g (CV 97%). The repeatability determined with the sample spiked at 6 pg WHO-TEQ per g showed a CV of 10% These results, shown in Chapter 2, clearly demonstrated that the reproducibility of the silica-CALUX procedure with samples containing more than 1 pg WHO-TEQ per g is relatively good, in particular regarding the fact that no internal standards could be used in the assay for correction of data for varying recoveries. The fact that the CV was much higher for the sample spiked at the lowest level, confirms the calculated limit of quantification of 1 pg WHO-TEQ per g fat.

The current tolerance limit for bovine milk in the EU is 3 pg WHO-TEQ per g fat, with an action limit of 2 pg WHO-TEQ per g fat. Therefore, the DR-CALUX® bioassay can be a useful pre-screening tool for selecting milk samples that may contain dioxin levels exceeding this tolerance. This was supported by the results obtained with 22 field samples, since all five samples exceeding the 2 pg WHO-TEQ per g fat concentration gave a higher response in the DR-CALUX® bioassay (Chapter 2). In addition to certain dioxins and PCBs, a number of polycyclic aromatic hydrocarbons (PAHs), like benzo(a)pyrene, have been shown to give a response in the DR-CALUX® bioassay [1, 2]. However, at low concentrations, the effect was only observed when cells were exposed for 4 hours. Furthermore, glucocorticoid hormones, in particular dexamethasone, and benzimidazole drugs have been shown to give a response in the assay [2]. However, regarding the relatively destructive nature of the 33% H_2SO_4 used in the procedure, it is not likely that these compounds would be present in the final extracts. Moreover, the use of the benzimidazole drugs is not allowed in dairy cows.

These data clearly demonstrate that the selection of specific test conditions, exposures of 24 h, in combination with the 33% H_2SO_4 clean-up procedure results in a specific test for the determination of dioxins and dioxin-like PCBs. The possible presence of benzimidazole drugs or glucocorticoids would result in a higher CALUX-response and could thus lead to false-suspect samples, which to a certain extent is acceptable for a qualitative screening method. False-negative results are limited and can easily be recognised by visual inspection of the cells (Chapter 1). It can be concluded that the DR-CALUX® bioassay is a very valuable tool, allowing the screening of relatively large sets of samples for the presence of unacceptable high levels of dioxins and dioxin-like PCBs.

This will result in a reduction of costs involved in the analysis of food for the presence of these compounds and will enable more intense monitoring programs.

1.2. Application of the DR-CALUX® bioassay

Following the successful optimisation and validation of the test for milk fat, the bioassay was first used at RIKILT in the food and feed area during the 1998 Brazilian citrus pulp incident. Increasing milk levels in German cows were traced back to the use of citrus pulp that had been mixed with contaminated lime [3]. Pulp samples of 5 gram were extracted and cleaned by the same procedure as used for the milk fat. A rapid comparison between CALUX and GC/MS data showed that the assay was capable of selecting the highly contaminated samples, using a cut-off value of 500 pg WHO-TEQ per kg. Most samples contained levels higher than this limit and required GC/MS confirmation. At the end of the crisis, the limit was officially set at 500 pg WHO-TEQ per kg, based on the detection limit of the GC/MS method. The test procedure was subsequently optimised and validated for animal feed.

Based on the consideration that an increased response is not necessarily caused by dioxins or dioxin-like PCBs, and that samples with an increased response would still have to be confirmed by GC/MS, it was decided to switch to a screening approach. This approach is based on the comparison of the response obtained with test samples with that of a reference sample, containing 400 pg WHO-TEQ per kg. Results obtained with 71 citrus pulp samples containing GC/MS determined levels between <250 and 6800 pg WHO-TEQ per kg showed that from 41 samples with a level below 500 pg WHO-TEQ per kg, 38 were negative and 3 (7%) showed an elevated response (false-suspect). From 30 samples with a level above 500 pg WHO-TEQ per kg, 27 (90%) showed an elevated response and no false-negatives were obtained. Three samples caused a cytotoxic effect, but following 5-fold dilution no toxic effect could be observed and the three samples were screened suspect. This shows that the test performs extremely well, even at these low residue limits [4].

In addition to the use of the DR-CALUX® bioassay during the 1998 Brazilian citrus pulp incident, the validated assay was also applied for food quality control during other incidents. Following the analysis of 100-1000 fold increased dioxin levels in three chicken feed, fat and egg samples by GC/MS in the spring of 1999, it soon became clear that a major food incident had happened: the Belgian dioxin incident. Due to poor traceability of the contaminated feed, many food samples became suspected and required testing. During the first month of the crisis, hundreds of in particular milk fat samples were screened with the bioassay. By the end of September, 4 months after the start of the incident, almost 1400 samples had been screened. Fat samples were screened by comparison with a milk fat sample containing 5 pg WHO-TEQ per g (2.7 pg TEQ dioxins and 2.3 pg TEQ non-ortho PCBs) and feed samples were screened by comparison with a citrus pulp sample containing 400 pg WHO-TEQ dioxins per kg [5, 6]. Starting in 2000 the CALUX-bioassay was introduced into monitoring programmes for dioxins in feed and feed ingredients in the Netherlands and later this was further extended to meat, eggs, fish, milk and other food samples.

During the German bakery waste incident in 2003, animal feed was contaminated with dioxins due to the use of waste wood for drying of the material. Besides Germany, the material was also shipped to the Netherlands. Levels up to 12 ng WHO-TEQ/kg have been detected, being about 15 times over the current limit of 0.75 ng WHO-TEQ/kg. A combined strategy of screening with the CALUX-bioassay and the HRGC/HRMS confirmatory method was used in the Netherlands to rapidly control the incident (Chapter 5). Pigs were contaminated by the incident but only to a very limited extent. Despite the rather low limits for pig meat, the DR-CALUX® bioassay, in combination with an extra acid pre-treatment of the fat samples, showed excellent performance, confirming once again the value of this bioassay (Chapter 5).

In 2000 the DR-CALUX® bioassay was accreditated at RIKILT for food and feed. Other food samples like egg and animal fat, and fish oil were shown to behave very similar to milk fat and extensive validation was not carried out. However, the suitability was demonstrated by inclusion of positive or spiked samples and in the meanwhile by a lot of historical datasets. In addition part of the negative samples are routinely checked by GC/MS to confirm the required low incidence of false-negative results. Other research groups validated the assay for blood samples from wild life species for high concentrations of dioxins and dioxin-like PCBs [7]. A special clean-up procedure was developed and validated for sediment, pore water and other environmental samples, allowing the use

of the assay for official testing of these sample types [8, 9]. Shown during the recent incidents with kaolinic clay (2004) and the contaminated HCl used for gelatine production (2006), the assay is still the best screening test for dioxins and dioxin-like PCBs.

2.1. Development of the RIKILT yeast Estrogen bioAssay (REA)

The second aim of the research in this thesis was to develop, validate and apply a new recombinant yeast screen to detect chemicals with an estrogenic mode of action in animal feed, urine and illegal preparations. The development of this recombinant yeast cell that stably expresses the human estrogen receptor α (hERα) and yeast enhanced green fluorescent protein (yEGFP) as a reporter protein in response to estrogens (REA), is described in Chapter 3. GFP is a protein that exhibits green fluorescence that can be measured directly [10]. This yEGFP can be measured much easier than the β-galactosidase, a reporter system frequently used by others in previous yeast based assays. The EC50 revealed by the REA was 0.5 nM for 17β-estradiol and was comparable with reported EC50 values for yeast estrogen bioassays that contain β-galactosidase as a reporter. The yEGFP assay can be performed completely in 96 well plates within 4 hours and does not need cell wall disruption nor does it need the addition of a substrate. This makes the test sensitive, rapid and convenient with high reproducibility and small variation. The robustness and ease of the yeast cells in combination with the qualities of yEGFP, ensure that the assay will be suited to be used as a high through put system (Chapter 3).

One of the potential applications of the REA is its use for detecting increased hormonal activity in samples of illegally treated animals. Therefore, it is essential to obtain data on the estrogenic potency of known and putative metabolites and to show the specificity of the assay for estrogenic compounds. In Chapter 4, the properties of the RIKILT yeast Estrogen bioAssay expressing the hERα (REA) were further studied by testing a series of estrogenic compounds. Furthermore, a similar assay was developed based on the stable expression of human estrogen receptor β (hERβ). When exposed to 17β-estradiol, the maximum transcriptional activity of the hERβ cytosensor was only about 40% of the activity observed with hERα, but the concentration where half-maximal activation is reached (EC50), was about 5 times lower. The relative estrogenic potencies (REP), defined as the ratio between the EC50 of 17β-estradiol and the EC50 of the compound, of the synthetic hormones dienestrol, hexestrol and especially mestranol were higher with ERα than with ERβ, while DES was slightly more potent with ERβ. The gestagens progesterone and medroxyprogesterone-acetate showed no response, whereas the androgen testosterone showed a very weak response and only at high concentrations. The anabolic agent 19-nortestosterone showed a clear dose-related response in the ERα based bioassay (REA), but not in the ERβ based system. This compound appears to have both androgenic and estrogenic properties, possibly explaining its excellent anabolic properties requiring both types of hormonal activity.

The isoflavones genistein, genistin, daidzein and daidzin, the coumestran coumestrol and the flavonoid naringenin were relatively more potent with ERβ than with ERα. Coumestrol and genistein were by far the most potent of these compounds with ERβ. However, 8-prenylnaringenin, a phytoestrogen present in hops, was relatively more potent with ERα than with ERβ and was actually the most potent phytoestrogen with ERα. Until now, comparison of 8-prenylnaringenin with ERα and ERβ was only performed in a receptor binding assay [11], revealing comparable binding activity to both receptor forms. In Chapter 4 we describe for the first time, that 8-prenylnaringen is more potent with ERα than with ERβ, using a yeast transcription activation assay. These observations are in agreement with effects described upon the intake of the female flowers of the hop plant, as they have long been used as a preservative. More recently, they have also been included in some herbal preparations for women for breast enhancement. Both applications indicate that 8-prenylnaringenin is an active agonist with ERα. The lignans enterolactone and enterodiol, thought to be responsible for estrogenic effects of flaxseed, did not show any response with either receptor type and are therefore characterised as being non-estrogenic in our yeast cytosensors.

Recently, the Interagency Coordinating Committee on the Validation of Alternative Methods, ICCVAM [12], presented a set of reference compounds for transcriptional activation assays and included the environmental pollutant 4-n-nonylphenol [CASRN 104-40-5] as a positive control. In our hands this compound did not show a response in either one of the yeast cytosensors. Others

however, have reported 4-n-nonylphenol to be active in these assay types [13, 14]. Thorough review of these studies showed that a technical mixture like the one available from Fluka (approximately 85 to 92.7% of branched isomers) or p-nonylphenol [CAS No. 84852-15-3] were used instead of the unbranched nonyl chain [CASRN 104-40-5]. When the 4-nonylphenol technical mixture of Fluka was used, it also showed a dose-response curve in our test with an EC50 of 100 nM. Chemical analysis with GC/MS confirmed the presence of many different congeners in this technical mixture of Fluka, but not of 4-n-nonylphenol, the aliphatic straight chain. From this, it can be concluded that 4-n-nonylphenol is not estrogenic and that the estrogenicity of the technical mixture is due to one or more isomers with a branched side-chain. A similar conclusion was presented by Pedersen et al. (1999), studying the induction of plasma vitellogenin in rainbow trout by linear and technical nonyl- and octylphenol [15]. Recently we tested many more reference compounds from the ICCVAM list and the results will be used in an overview about the value of yeast bioassays. This will include a critical review of the chemicals presented by ICCVAM as positive and negative controls.

A number of metabolites of 17β-estradiol and estrone showed a clear response, but in general at concentrations that were a factor 5 to 105 higher then those of their parent compounds (Chapter 4). The presented data also show that for testing of e.g. urine of calves a deconjugation step is a requirement for generating the compounds actually active as estrogen agonists and, thus, generating sensitivity. As 1% deconjugation of the 17β-estradiol conjugates would already result in a REP of 0.01 and as the REPs of these conjugates are much lower than 0.01 and because dose-response curves after a relatively short exposure period of 4 h were similar to those after 24 h, it is most likely that the yeast cells themselves are not or hardly able to deconjugate these compounds. For the same reasons the estrogenic activity of 17β-estradiol-3-benzoate, with a REP of 0.009, suggests similar poor metabolic conversion of the ester.

Together the data presented in Chapters 3 and 4 demonstrate that the REA can be extremely valuable for the screening of estrogenic activity in animal feed, urine, illegal preparations and tissue or plasma samples, as the REA showed clear dose-response curves when exposed to estrogenic compounds. These hERα (REA) and hERβ based yeast bioassays are not only very sensitive, but also very rapid, convenient, reproducible and most likely more robust than similar bioassays based on mammalian cell-lines. Since good dose-response curves can be obtained after only 4 h of exposure, the often questioned permeability of the yeast cell wall does not seem to be an obstacle in our yeast estrogen bioassays.

2.2. Validation and application of the REA

In Chapter 6 the Rikilt yeast Estrogen bioAssay stably expressing human estrogen receptor α (REA) was validated as a qualitative screening method for the determination of estrogenic activity in calf urine. This validation was performed according to EC Decision 2002/657, which prescribes the determination of the detection capability (CCβ), the specificity and the stability. To determine these performance characteristics, twenty blank urine samples of 19 week old calves were collected and spiked with 17β-estradiol (E2β) at 1 ng ml-1, diethylstilbestrol (DES) at 1 ng ml-1, 17α-ethynylestradiol (EE2) at 1 ng ml-1, α-zearalanol at 50 ng ml-1 or mestranol at 10 ng ml-1. Following enzymatic deconjugation and solid phase extraction, 100 µl equivalents of these blank and spiked urine samples were screened for estrogenic activity in a 96 well plate using the REA. All of these low estrogen spiked urine samples could be distinguished from the blank samples as all spiked samples gave a signal above the determined decision limit CCα and the mean responses of the spiked samples were higher than the determined detection capability CCβ. As the CCβ criterion was met, these spiked samples have a lower than 5% probability to be classified as a false-negative. The specificity of the method was determined with blank urine samples spiked with a high dose of testosterone or progesterone (1000 ng ml-1). No response to these substances was detected in the REA. There was also no interference of a high dose of testosterone or progesterone on the response of a low dose of the estrogens. Stability of urine samples was checked with spiked urine samples that were kept frozen for up to 90 days, showing that urine samples could be stored at -20 °C for up to 60 days without changing the screening result of the assay. At present this method has been in routine use at RIKILT for more than two years. Figure 1 shows a control chart, showing the decision limit CCα and the corrected responses for the reagent blank, blank urine control, spiked reagent blank and the spiked urine control. Only twice a blank urine control was found suspect (less than 4%) and spiked controls

were always found suspect. In May 2005 the REA acquired an ISO 17025 accreditation status in the Netherlands for this urine matrix.

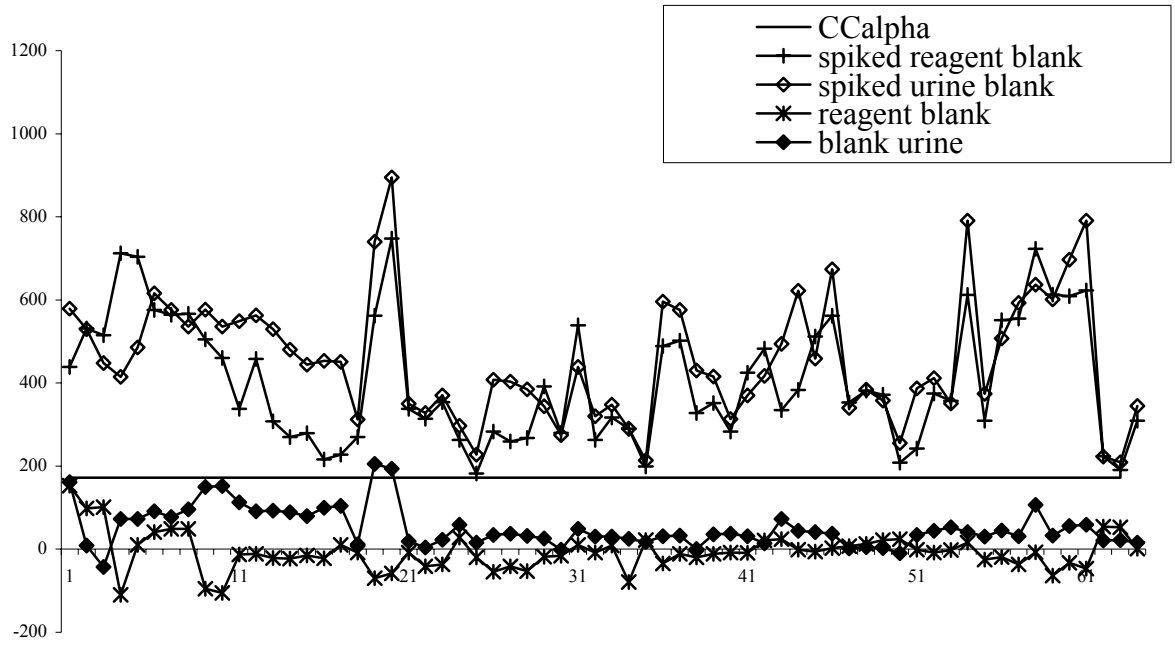

Figure 1. Long-term response (n=65, in about 2 years) in the REA of the reagent blank, the reagent blank spiked with 1 ng 17β-estradiol per ml, the blank urine sample and the blank urine sample spiked with 1 ng 17β-estradiol per ml.

In another study, 126 calf urine samples, derived from untreated calves, were screened with the REA and a comparison was made with results obtained with a gas chromatography tandem mass spectrometry confirmatory analysis method16. The results of both methods are summarised in Figure 2. According to the GC/MS/MS method 71 out of the 126 calf urine samples were compliant for estrogens, i.e. all estrogens included in the instrumental multi-residue method were below 1 ng per ml. In the REA screening 67 out of 126 were screened compliant for estrogen activity. Only 4 out of the 71 (5.6%) calf urine samples found compliant in GC/MS/MS were screened false suspect in the REA screening method, assuming that application of the mass spectrometric method is considered beyond reasonable doubt. In the 55 samples found non-compliant with the GC/MS/MS method, the natural estrogen 17α-estradiol was identified, occasionally accompanied by estrone. The presence of these natural estrogens in urine from individual calves is not unexpected when they reach the age of 20-28 weeks. The bioassay was less sensitive than GC/MS/MS for the relatively weak estrogenic compound 17α-estradiol, in accordance with expectations (REP of 0.09). It was therefore not surprising that the 15 samples containing 1 ng 17α-estradiol per ml, i.e. 0.09 ng “17β-estradiol equivalents” per ml, were screened negative in the REA. The REA results of the other non-compliant urine samples under routine conditions, containing 2, 3, 4 and 6 ng 17α-estradiol per ml were also as predicted, taking into account the relative estrogenicity of the natural estrogens 17α-estradiol and estrone versus 17β-estradiol, respectively a REP of 0.09 and 0.2. Only one urine sample at the 10 ng 17α-estradiol per ml level, that also contained a low level of estrone (about 1 ng per ml), should have triggered the bioassay as it contained a total of 1.1 ng “17β-estradiol equivalents” per ml. So, for once the bioassay gave a real false-compliant result (1 out of 126 samples). However, this sample was re-analysed later in 6-fold and was always screened suspect.

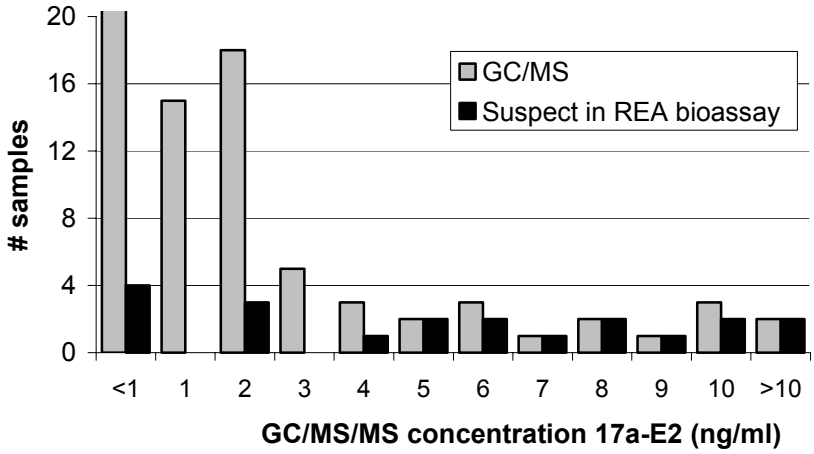

Figure 2. Estrogen screening in calf urine samples, comparison of the results obtained by the REA and an established GC/MS/MS instrumental analysis method [16].

In Chapter 7 the REA was validated for the screening of estrogenic activity in animal feed. In addition two incidents with animal feed, in which the RIKILT yeast estrogen bioassay was responsible for the detection of estrogenic substances, were presented. The validation was performed according to EC Decision 2002/657. As in the case of the urine samples, twenty blank animal feed samples, including milk replacers and wet and dry feed samples, were spiked with 17β-estradiol (E2β) at 5 ng g-1, 17α-ethynylestradiol (EE2) at 5 ng g-1, diethylstilbestrol (DES) at 10 ng g-1, zearalenone at 1.25 µg g-1 or equol at 200 µg g-1. All of these blank and low estrogen spiked feed samples fulfilled the CCα and CCβ criterions, meaning that all 20 blank feed samples gave a signal below the determined decision limit CCα and were thus classified as compliant and at least 19 out of the 20 spiked samples gave a signal above this CCα (β=5%) and were thus classified as suspect. High levels of the androgen 17β-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 demonstrated that the bioassay was 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 described clean-up/yeast estrogen bioassay procedure was 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 through-put 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 was enough to be processed. The method acquired an ISO 17025 accreditation status in the Netherlands for feed in December 2005. It is in routine use at RIKILT for more than one year now and it showed good robustness: feed extracts were almost never cytotoxic (less than 1% of the samples). Reagent blank and blank feed controls were always found compliant and the spiked reagent blank and spiked feed controls were always screened suspect (Chapter 7). The examples of the MPA-incident with wet pig feed and the fish feed, described in Chapter 7, demonstrate the applicability of the bioassay method as an early warning system for pharmaceutical waste and hormone use respectively. This is the first successful example of a developed, validated and applied bioassay for the screening of hormonal substances in feed.

3. Future developments

3.1. DR-CALUX®

Future investigations will have to reveal which compounds may actually interfere with the test result and whether these compounds should be included in the TEQ-principle and legislation.

3.2. Yeast based assays for the detection of hormonal activities

The robustness, sensitivity, easy handling and good reproducibility of the REA bioassay, made us decide to develop similar bioassays for androgens, gestagens and glucocorticoids as well. Recently, the RIKILT yeast Androgen bioAssay (RAA) was developed [17]. When exposed to 17β-testosterone, the concentration where half-maximal activation is reached (EC50) was about 50 nM. The relative androgenic potencies (RAP), defined as the ratio between the EC50 of 17β-testosterone and the EC50 of the compound, of 5α-dihydrotestosterone, 19-nor-17β-testosterone, R 1881, tetrahydrogestrinone and 17β-boldenone are 2.3, 1.2, 0.4, 0.4 and 0.3 respectively. The steroids 17α-estradiol, estrone, 17α-ethynylestradiol, progesterone, corticosterone and dexamethasone showed no response. This demonstrates that this new yeast androgen assay is sensitive and very specific, i.e. there is no cross-talk from other hormone activities as is the case in mammalian cell lines that express more receptors than the androgen receptor only. Moreover, studies with the eight isomers of androstanediol, the four isomers of dihydrotestosterone and the four isomers of androsterone showed that especially the conformation, α or β, at position 5 and 17 are important for the activity of the compound.

In another study the feasibility of this new RAA in combination with mass spectrometric identification was investigated for trace analysis of designer steroids in urine [18]. Human urine samples were spiked with the designer anabolic steroid tetrahydrogestrinone (THG). Following enzymatic deconjugation and generic solid-phase extraction, samples were analysed by gradient LC with effluent splitting toward two identical 96-well fraction collectors. One plate was used for androgen bioactivity detection using the new robust Rikilt yeast Androgen bioAssay (RAA) yielding a bioactivity chromatogram featuring a 20 sec time resolution. Figure 3 shows the androgen bioactivity chromatograms of a reagent blank sample spiked with 17β-boldenone (well#11), 17β-testosterone (well#24) and THG (well# 33/34) (15 ng/ml each), an adult female urine sample and an adult female urine sample spiked with THG (15 ng/ml). The diagram clearly shows the androgenic activity in well 33/34 from THG in the spiked female urine sample. Analysing the corresponding well number in the duplicate well plate by LC/QTOFMS resulted in a [M+H]+ ion at m/z 313 and fragments with element compositions18 that are in full agreement with the recently proposed fragmentation scheme of THG ($C_{21}H_{28}O_2$). In the near future this RAA bioassay will be validated for animal feed and calf urine and used to detect androgens and designer steroids in supplements used for veterinary abuse and sports doping. Moreover, yeast based assays will be developed for gestragens and glucocorticosteroids.

4. General conclusion

The receptor based bioassays described in this thesis, the DR-CALUX® and the REA, and even the above described RAA, prove that receptor based transcription activation assays are valid and cheap tools for the screening of dioxins, estrogens and androgens in food and feed.

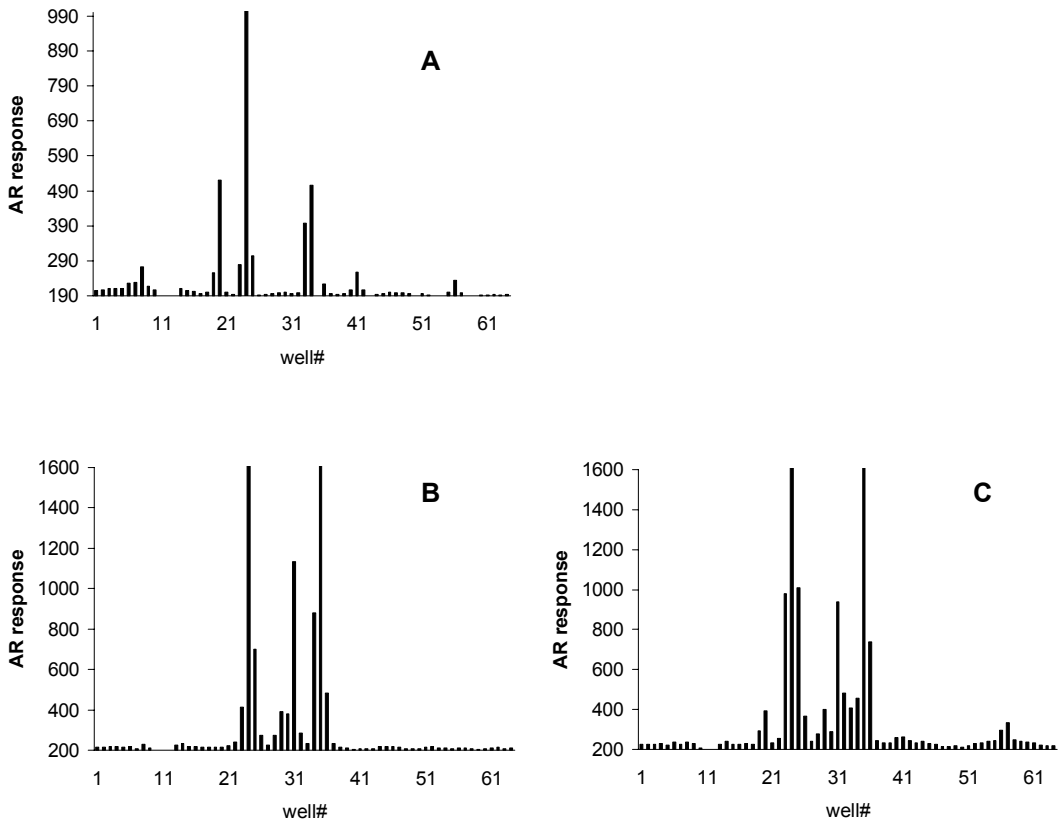

Figure 3. Androgen bioactivity chromatograms obtained with the RAA of HPLC fractionation of (a) a reagent blank sample spiked with 17β-boldenone, 17β-testosterone and THG (15 ng/ml each), (b) an adult female urine sample and (c) the same adult female urine sample spiked with 15 ng THG per ml [18].

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Samenvatting

SAMENVATTING

Dit proefschrift beschrijft de ontwikkeling, validatie en routinematige toepassing van bioassays voor de detectie van dioxinen en oestrogenen in de voedselketen. Na de introductie in Hoofdstuk 1, beschrijft Hoofdstuk 2 de validatie van de DR-CALUX® bioassay voor de detectie van dioxinen en PCBs in melk. Deze test is gebaseerd op genetisch gemodificeerde levercellen van de rat. Deze cellen produceren een lichtgevend eiwit als ze worden blootgesteld aan stoffen met een dioxineachtige werking. Hoofstuk 3 en 4 beschrijven de ontwikkeling van de RIKILT Estrogenen bioAssay (REA). Deze test is gebaseerd op een genetisch gemodificeerde gistcel die de humane oestrogeen receptor α tot expressie brengt. Deze gistcellen fluoresceren licht als ze worden blootgesteld aan stoffen met een oestrogene werking. Hoofdstuk 4 beschrijft bovendien de ontwikkeling van een gist bioassay die gebaseerd is op het tot expressie brengen van de humane oestrogeen receptor en produceren ook het groene fluorescerende eiwit als ze worden blootgesteld aan oestrogenen. De oestrogeen receptor β speelt mogelijk een belangrijke rol bij de effecten van sommige planthormonen, de zgn. phytoestrogenen.

Hoofdstuk 5 beschrijft de toepassing van de DR-CALUX® bioassay tijdens een van de recente dioxine-incidenten, namelijk het Duitse dioxine-incident met bakkersafval in 2003. Afval van bakkerijen werd gedroogd en gebruikt voor het maken van diervoeders. Gebleken is dat het verbranden van afvalhout voor het drogen van het bakkersafval tot de besmetting leidde. Hoodstuk 6 en 7 beschrijven de validatie en toepassing van de RIKILT oestrogenen bioassay (REA) voor het screenen van oestrogeen activiteit in urine van kalveren en diervoeder. Hoofdstuk 7 laat tevens een tweetal voorbeelden zien waarin het gebruik van de REA test leidde tot de detectie van oestrogeen activiteit in diervoeders. Het eerste voorbeeld was het MPA incident in 2002. Voeders gaven een verdacht signaal in de REA test, maar MPA was niet verantwoordelijk voor de uitslag in de test. Door fractionering van het monster en het testen van de fracties met de REA test werd uiteindelijk met behulp van de instrumentele analysetechniek (GC/MS) de verantwoordelijke stof, 17β-estradiol, geïdentificeerd. In 2004 werd met behulp van de REA op soortgelijke wijze het gebruik van 17β-estradiol in visvoeders aangetoond.

1.1. Optimalisatie en validatie van de DR-CALUX® bioassay

Een speciale cellijn voor de detectie van dioxinen is door de Vakgroep Toxicologie van Wageningen Universiteit ontwikkeld in een gezamenlijk project met de Universiteit van Californië in Davis en het RIKILT. In Hoofdstuk 2 van dit proefschrift is deze DR-CALUX® bioassay getest en geoptimaliseerd voor de detectie van lage gehalten aan dioxinen in koemelk. De resultaten toonden aan dat deze test zeer gevoelig is voor 2,3,7,8-gesubstitueerde dioxinen en dioxineachtige PCBs. Omdat de cellijn echter niet uitsluitend gevoelig is voor dioxinen en PCBs, werd een procedure ontworpen om andere stoffen uit de matrix te breken verwijderen (Hoofdstuk 1 en 2). Melkvet werd gezuiverd over een silica kolom die voor 33% bestond uit zwavelzuur (H2SO4). Om de methode te valideren werd schoon melkvet op verschillende niveaus gecontamineerd met een mengsel van 17 verschillende 2,3,7,8-gesubstitueerde polychloor dibenzo-p-dioxinen (PCDD) en dibenzofuranen (PCDF). De huidige dioxinenorm voor melk in de Europese Unie is 3x10-12. gram WHO-TEQ aan dioxinen per gram vet en de actielimiet is 2x10-12 gram TEQ aan dioxinen per gram vet. De resultaten van de validatiestudie en de resultaten met 22 praktijkmonsters toonden duidelijk aan dat monsters die meer dan 2x10-12 gram TEQ per gram vet bevatten, een verhoogd signaal in de DR-CALUX® bioassay test gaven. Dit vormde een belangrijke ondersteuning voor de toepassing van de test.

1.2. Toepassing van de DR-CALUX® bioassay

Na de succesvolle optimalisatie en validatie van de DR-CALUX® test voor melkvet, werd de methode voor het eerst toegepast op voedsel en voer tijdens het Braziliaanse citruspulp incident in 1998. In Duitsland namen de gehalten aan dioxinen in melk toe door het voeren van citruspulp dat was gemengd met gecontamineerde kalk. De test bleek geschikt te zijn om positieve monsters te scheiden van onbesmette monsters.

In de lente van 1999 werd met GC/MS analyse, een combinatie van gaschromatografie en massaspectroscopie, in een drietal monsters, kippenvoer, kippenvlees en ei, een 100-voudige overschrijding van de dioxinenorm gemeten: de Belgische dioxine crisis. Doordat het met een PCB-olie besmette voer en de producten afkomstig van besmette dieren, zolang na het incident, slecht te

traceren waren, werden veel producten verdacht en die moesten dus getest worden. Op het RIKILT werden tijdens de eerste maand enkele honderden, vooral melkvetmonsters, gescreend met de DR-CALUX® test. Vier maanden na het incident waren ruim 1300 monsters gescreend. Vetten werden gescreend en vergeleken met een referentie melkvet dat 5x10-12 gram TEQ per gram vet bevatte. Die 5 pg (=5X10-12 gram) bestond uit 2,7 pg TEQ aan dioxinen en 2.3 pg TEQ aan non-ortho PCBs. Diervoeders werden gescreend en vergeleken met een referentie citruspulp die 400 pg TEQ per kg aan dioxinen bevatte. Sinds 2000 wordt in Nederland de CALUX-bioassay op het RIKILT ingezet in diverse monitoring programma's van het ministerie van LNV en VWS. En daarna volgden nog een aantal incidenten waarbij de test werd ingezet of zelfs leidde tot de ontdekking van de besmetting.

Het dioxine-incident met bakkersafval in Duitsland in 2003, waarbij diervoer was besmet met dioxinen, is een ander voorbeeld van een succesvolle toepassing van de CALUX-bioassay (Hoofdstuk 5). Door het verbranden van gecontamineerd afvalhout voor het drogen van het bakkersafval werd het voer vervuild met dioxinen. Het besmette broodmeel werd ook op diverse Nederlandse bedrijven geleverd en gehalten tot 12x10-9 gram TEQ per kg zijn waargenomen. Dit is ongeveer 15 maal zoveel als de toegestane norm van 0,75x10-9 gram TEQ/kg. Om het incident snel onder controle te krijgen, werd op het RIKILT een gecombineerde strategie van screening met de CALUX-bioassay en confirmatie met de GC/HRMS referentiemethode gebruikt. Varkens waren besmet, maar ondanks de lage norm voor varkensvlees was het afkeuringpercentage erg laag. Bovendien bleek een extra zure voorbehandeling van de vetmonsters noodzakelijk vanwege de lage norm van 1 pg TEQ per gram vet voor varkensvlees. Met deze nieuwe clean-up procedure bleek de test uitstekend te voldoen en nagenoeg dezelfde resultaten op te leveren als de HRGC/HRMS referentiemethode (Hoofdstuk 5).
Ook tijdens de meest recente incidenten, in 2004 met besmette klei uit Duitsland die werd gebruikt voor het selecteren van aardappelen voor het maken van friet en in 2006 met besmet zoutzuur (HCl) dat werd gebruikt voor de productie van gelatine in België, is gebleken dat de assay nog steeds een goede screeningstest is voor de detectie van dioxinen en dioxineachtige PCBs in voedsel en voer.

2.1. Ontwikkeling van de RIKILT gist Estrogenen bioAssay (REA)

Het tweede doel van het onderzoek in dit proefschrift was om een nieuwe op gist gebaseerde test te ontwikkelen voor het detecteren van stoffen met een oestrogeen werking. Bestaande assays met gist of zoogdiercellen bleken een aantal nadelen te hebben, waardoor ze minder geschikt waren voor het screenen van urine en voer. Dit betreft onder meer de stabiliteit van de test, het reportereiwit en de bepaling daarvan, de specificiteit en de gevoeligheid voor andere stoffen in de extracten. De ontwikkeling van de recombinant gistcel, die de humane oestrogeen receptor alfa (hERα) tot expressie brengt en een groen fluorescerend eiwit (yEGFP) product in reactie op stoffen met een oestrogene werking, is beschreven in Hoofdstuk 3.

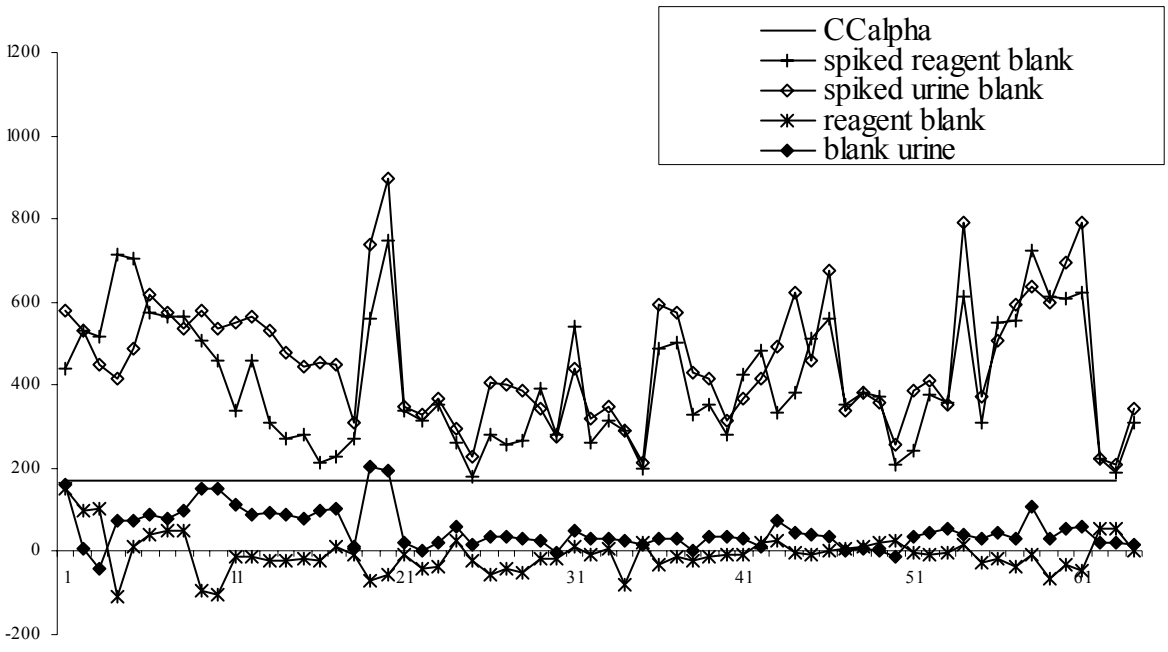
Mogelijke toepassingen van deze RIKILT gist Estrogenen bioAssay (REA) zijn: om een toename in hormoonactiviteit vast te stellen in urine monsters van illegaal behandelde dieren, het testen van voeders en het testen van illegale preparaten. Het is daarom van belang om te weten hoe de REA-bioassay reageert op bekende oestrogenen en metabolieten om zo de gevoeligheid en specificiteit van de assay voor oestrogenen te bepalen. Om die reden zijn in Hoofdstuk 4 een serie oestrogenen getest in de REA-bioassay. In Hoofdstuk 4 wordt bovendien de ontwikkeling van een gist bioassay beschreven die gebaseerd is op de expressie van de humane oestrogeen receptor bèta (hERβ). Vergeleken met de hERα bioassay (REA), is de maximale respons met 17β-estradiol in de hERβ bioassay slechts 40%, maar de concentratie waarbij de helft van de maximale respons wordt bereikt (EC50) is in de hERβ bioassay ongeveer 5 keer lager. De relatieve oestrogene potenties (REP), gedefinieerd als de verhouding tussen de EC50 van 17β-estradiol en de EC50 van een component, zijn voor de synthetische hormonen dienestrol, hexestrol en mestranol hoger in de REA (hERα), terwijl DES een beetje potenter is in de hERβ bioassay. De gestagenen progesteron en medroxyprogesteron-acetaat, beter bekend als MPA, geven geen signaal en testosteron geeft alleen bij zeer hoge concentraties een zwak signaal. Het anabole steroïd 19-nortestosteron geeft een duidelijke dosis-afhankelijke respons in the REA, maar niet in de hERβ bioassay. Het lijkt erop dat deze stof zowel een androgene als ook een oestrogeen activiteit heeft. Dat zou ook verklaren waarom deze stof zulke goede anabole eigenschappen heeft (Hoofdstuk 4), omdat voor een sterk groeibevorderend effect beide activiteiten nodig zijn.

De humane oestrogeen receptor α komt voornamelijk tot expressie in de borstklier en de baarmoeder en de humaan oestrogeen receptor β met name in botten, het hart en vaatstelsel, het urinewegstelsel en het centrale zenuw stelsel. In overeenstemming met de literatuur bleek dat de phytoestrogenen genistein, genistin, daidzein, daidzin, coumestrol en naringenin relatief potenter zijn

met de hERβ-gist dan met de hERα-gist. Voor 8-prenylnaringenin, een phytoestrogeen uit hop, was dit omgekeerd. In Hoofdstuk 4 van dit proefschrift wordt deze specifieke hERα activiteit van 8-prenylnaringenin voor het eerst aangetoond. Deze bevindingen komen overeen met effecten beschreven op vrouwelijke plukkers van hop, die tijdelijk onvruchtbaar werden. Hopextracten werden dan ook lange tijd gebruikt als anticonceptiemiddel, maar de actieve stof was niet bekend. Sinds een aantal jaren wordt deze stof toegepast in kruidenmengsels die worden verkocht voor borstvergroting. Beide toepassingen duiden erop dat 8-prenylnaringenin een sterke agonist is op de hERα.
De gegevens, die zijn gepresenteerd in Hoofdstuk 3 en 4 van dit proefschrift, maken duidelijk dat de REA een waardevolle bioassay is. De REA is gevoelig, snel, gemakkelijk en reproduceerbaar.

2.2. Validatie en toepassing van de REA

In Hoofstuk 6 is de REA-bioassay gevalideerd als een kwalitatieve screeningsmethode voor het detecteren van oestrogene activiteit in urine van kalveren. Deze validatie is uitgevoerd volgens de richtlijnen van de EU zoals die zijn vastgelegd in EC Decision 2002/657. Twintig blanco urinemonsters van kalfjes van 19 weken oud werden besmet met 17β-estradiol (E2), diethylstilbestrol (DES), 17α-ethynylestradiol (EE2) (allen met 1 ng per ml), α-zearalanol (50 ng per ml) of mestranol (10 ng per ml). Na enzymatische deconjugatie en kolomzuivering, werden equivalenten van 0,1 ml van deze monsters in een 96-wells plaat overgebracht en met de REA-bioassay gescreend op oestrogene activiteit. De 20 blanco monsters gaven allemaal een signaal dat lager was dan de vastgestelde afkeurgrens CCα en alle besmette monsters gaven een hoger signaal dan de afkeurgrens CCα. De kans op vals-negatieve uitslagen is daardoor kleiner dan 5%, waardoor de test voldoet aan de eisen. De specificiteit van de methode is bepaald door blanco monsters te besmetten met een hoge dosis 17β-testosteron of progesteron (1000 ng per ml). Deze monsters gaven geen signaal in de REA. Er was ook geen interferentie van deze stoffen op de met een lage dosis aan oestrogenen besmette monsters. De stabiliteitsstudie toonde aan dat de urinemonsters 90 dagen konden worden bewaard in de vriezer (-20°C). Op het RIKILT wordt de methode inmiddels al ruim twee jaar routinematig toegepast. Figuur 1 toont een controle kaart die de afkeurgrens CCα en de respons van een blanco urine, blanco reagens, besmette blanco reagens en een besmette blanco urine laat zien. Uit de resultaten blijkt duidelijk dat de positieve controles altijd als verdacht uit de test komen. Slechts 2 keer kwam een blanco urine als verdacht uit de test (<4%). In mei 2005 heeft deze REA test een ISO 17025 accreditatie gekregen voor deze urine matrix.



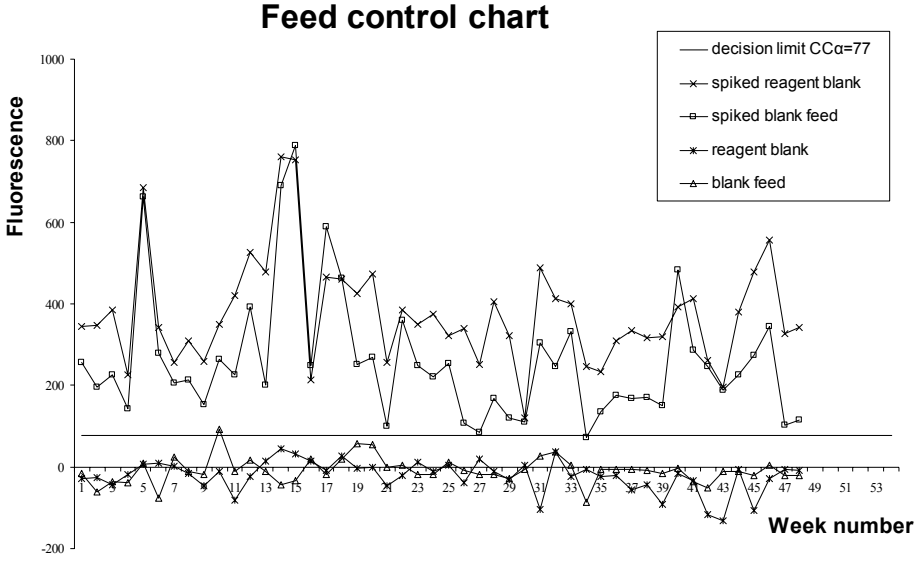
Figuur 1. Respons in de REA test (n=65) van een blanco urine, blanco reagens, de blanco urine besmet met 1 ng 17β-estradiol per ml en de blanco reagens besmet met 1 ng 17β-estradiol per ml.

In het onderzoek beschreven in hoofdstuk 7 van dit proefschrift is de REA gevalideerd als een kwalitatieve screeningsmethode voor het detecteren van oestrogene activiteit in diervoeders. Net als voor de urine, werden 20 blanco voeders besmet met 17β-estradiol (E2), 17α-ethynylestradiol (EE2) (beiden met 5 ng per g), diethylstilbestrol (DES) (10 ng per g), zearalenon (1250 ng per g) of equol (200 μg per g). Na extractie en kolomzuivering werd een equivalent van 67 mg voer in een 96-wells plaat met de REA gescreend op oestrogene activiteit. Alle blanco en besmette monsters voldeden aan de criteria. Dat betekent dat alle blanco monsters een signaal gaven dat lager was dan de vastgestelde afkeurgrens CCα en dat tenminste 19 van de 20 besmette monsters een signaal gaven dat hoger was dan de afkeurgrens CCα. Hierdoor is de kans op vals-negatieve uitslagen kleiner dan 5%.

Ook voor voer is de methode niet alleen zeer gevoelig, maar ook specifiek, want voeders besmet met 1000 ng 17β-testosteron of progesteron gaven geen verdacht signaal. Het signaal was dus lager dan de vastgestelde CCα. Er was ook geen interferentie van deze stoffen. Voeders konden gemakkelijk gedurende 98 dagen worden opgeslagen zonder dat het screeningsresultaat (verdacht of negatief) werd beïnvloed. Dit toont aan dat de methode ook robuust is.

Hoofdstuk 7 laat tevens een tweetal voorbeelden zien waarin het gebruik van de REA test leidde tot de detectie van oestrogene activiteit in diervoeders en de identificatie van de verantwoordelijke stof. Het eerste voorbeeld was het MPA incident in 2002. Farmaceutisch afval met medroxyprogesteron-acetaat (MPA) kwam terecht in brijvoeders voor varkens. Voeders gaven een verdacht signaal in de REA-test, maar MPA bleek niet verantwoordelijk voor deze uitslag. Door fractionering van het monster en het testen van de fracties met de REA-test werd uiteindelijk de verantwoordelijke stof, 17β-estradiol, met massaspectroscopie geïdentificeerd. Deze stof was als bijproduct aanwezig in het farmaceutische afval. In 2004 werd met behulp van de REA op soortgelijke wijze het gebruik van 17β-estradiol in visvoeders aangetoond.

Voor voeders wordt de methode op het RIKILT inmiddels al meer dan een jaar routinematig toegepast. Figuur 2 toont een controle kaart die de afkeurgrens CCα en de respons van een blanco voer, blanco reagens, besmet blanco reagens en een besmet blanco voer laat zien. Uit de resultaten blijkt duidelijk dat de positieve controles altijd als verdacht uit de test komen en dat de negatieve controles altijd als negatief uit de test komen. Dit is de eerste bioassay die is gevalideerd en wordt toegepast voor het screenen van oestrogenen in diervoeders. In december 2005 heeft deze REA test een ISO 17025 accreditatie gekregen voor het screenen van diervoeders.



Figuur 2. Respons in de REA test (n=48) van een blanco voer, blanco reagens, het blanco voer besmet met 5 ng 17β-estradiol per g en de blanco reagens besmet met 5 ng 17β-estradiol per g.

De REA-test is de eerste en enige geaccrediteerde en toegepaste bioassay methode voor de bepaling van oestrogene activiteit in urine van kalveren en diervoeders. Dankzij de goede gevoeligheid van de REA-bioassay zijn 1 ml urine en 1 g voer al voldoende om een positief monster op te sporen. De procedure voor de opwerking van de monsters is eenvoudig en de REA-bioassay is naast gevoelig, ook nog snel, gemakkelijk, reproduceerbaar en robuust. Deze eigenschappen zorgen ervoor dat het RIKILT beschikt over een high-throughput methode voor het screenen van oestrogene activiteit in urine, voeders en illegale preparaten.

3. Toekomstige ontwikkelingen

3.1. DR-CALUX

Verdere verlaging van de normen voor dioxinen en aparte normen voor dioxineachtige PCBs betekenen dat de assay eigenlijk gevoeliger zou moeten worden. Alternatief is uiteraard het in bewerking nemen van grotere monsterhoeveelheden, maar dat geeft problemen met de snelle opwerking van grote aantallen monsters. Ook zou de reproduceerbaarheid van de clean-up verbeterd moeten worden, omdat de huidige clean-up de nodige ervaring vereist. In het kader van het opsporen van nieuwe agonisten zou additioneel onderzoek moeten uitwijzen welke stoffen er kunnen “interfereren” met het screeningsresultaat.

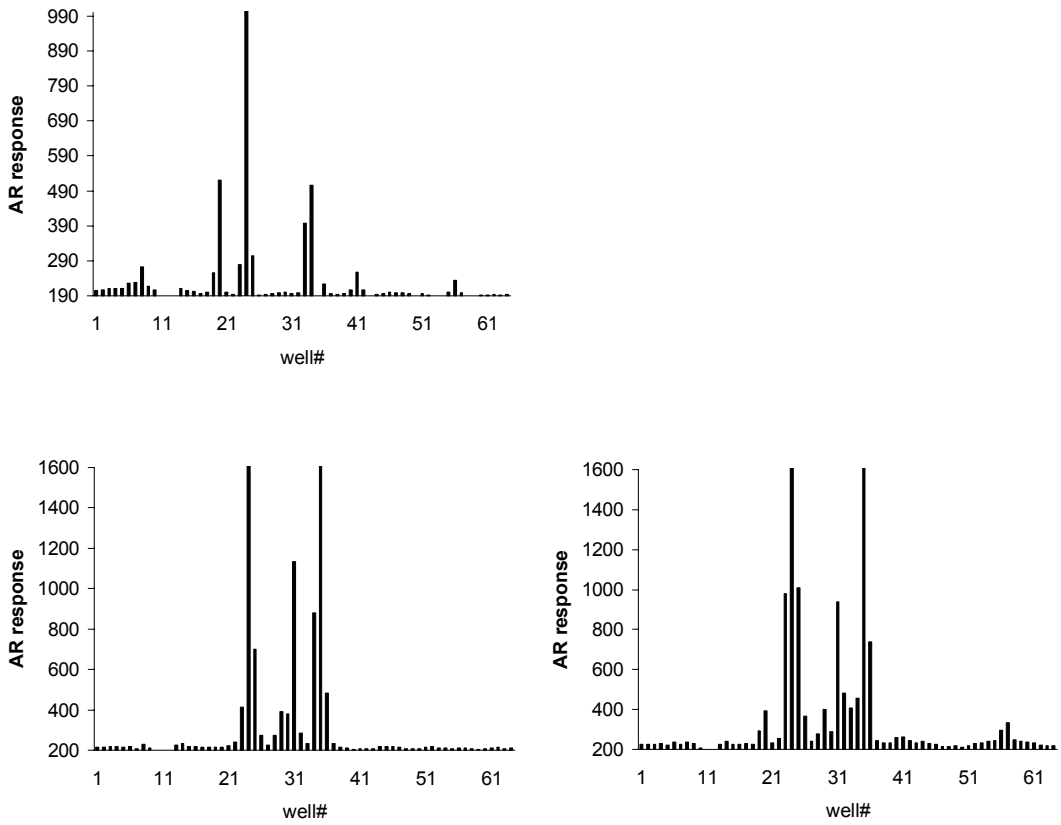
3.2. Op gist gebaseerde bioassays voor de detectie van hormonale activiteiten

De gevoeligheid, eenvoud, robuustheid, reproduceerbaarheid en het succes van de REA-bioassay hebben er toe geleid, dat het RIKILT vergelijkbare, op gist gebaseerde bioassays, gaat ontwikkelen voor de detectie van androgenen, progesteronachtigen en glucocorticosteroïden. De RIKILT yeast Androgen bioAssay (RAA) is recent ontwikkeld. Deze nieuwe androgene bioassay is gevoelig gebleken voor 17β-testosteron, 5α-dihydrotestosteron, 19-nor-testosteron, methyltrienolone (R 1881), 17β-boldenon, 17β-trenbolon en tetrahydrogestrinon, beter bekend als THG. De EC50 van deze stoffen ligt in de range van 20 tot 200 nM. In tegenstelling tot androgene bioassays die zijn gebaseerd op humane cellijnen, is de RAA-bioassay wel specifiek voor androgenen (geen crosstalk met andere type hormonale activiteiten). Stoffen als 17α-estradiol, estrone, 17α-ethynylestradiol, progesteron, corticosteron en dexamethason geven geen respons in onze RAA-bioassay.

In een proef met humane urines die waren besmet met het synthetische steroïd THG, bleek duidelijk dat deze nieuwe androgene bioassay zeer geschikt is voor het opsporen van onbekende androgenen. Figuur 3 laat het bio-activiteitschromatogram zien van een blanco reagens besmet met 17β-boldenon (well#11), 17β-testosteron (well#24) en THG (well#33/34) (A: 15 ng/ml van elk), een vrouwelijk urinemonster (B) en een vrouwelijk urinemonster besmet met THG (C: 15 ng/ml). Het diagram laat duidelijk de androgene activiteit in well 33/34 van THG in het besmette vrouwelijke urinemonster zien. Deze RAA-bioassay zal dit jaar worden gevalideerd voor diervoeders en kalverurine en in de toekomst worden gebruikt voor het opsporen van designer steroïden in supplementen voor dierlijk gebruik en sportdoping.

4. Algemene conclusie

De ervaringen opgedaan met de DR-CALUX® en REA-bioassays die worden beschreven in dit proefschrift, en ook de hierboven beschreven RAA-bioassay, bewijzen dat receptorgen-assays van grote waarde zijn voor het screenen van voedsel en voeders op stoffen als dioxinen, estrogenen en androgenen..Een groot voordeel daarbij is, dat de detectie van stoffen op basis van hun activiteit plaats vindt. Hierdoor kunnen nieuwe stoffen snel en eenvoudig worden opgespoord, maar nog belangrijker is dat hun aanwezigheid kan worden uitgesloten, ook als de structuur van de stoffen nog niet bekend is.



Figuur 3. Androgeen bio-activiteitschromatogram verkregen met de RIKILT gist Androgenen bioAssay (RAA) van: (A) een blanco reagens besmet met 17β-boldenon, 17β-testosteron en THG (15 ng/ml van elk), (B) een vrouwelijk urinemonster en (C) een vrouwelijk urinemonster besmet met 15 ng THG per ml.

Curriculum Vitae

Toine Frank Henk Bovee was born in Eindhoven, the Netherlands on April 7, 1966. In 1984 he finished secondary school at the Elzendaalcollege in Boxmeer (Athenaeum β). Starting in September 1984, he studied Molecular Sciences at the Agricultural University of Wageningen and graduated in August 1990. He chose the chemical-biological orientation and a biotechnological profile with accents on biochemistry and molecular biology. His first research subject was carried out at the Department of Biochemistry of Wageningen University, where lyis of different bacteria in an organic solvent was studied. The second research subject at the Department of Process Engineering was dedicated to the uptake of α-amylase in reversed micelles. The effects of fatty acids on the structure of blood vessels' elastine was his third topic at the Department of Food Physics. From 1991 to 1993 he worked at Organon International in Oss and at Organon Teknika in Boxtel. At Organon International he was involved in quality control of tabletted hormones and in freeze drying techniques to stabilise active compounds. At Organon Teknika he development new HIV-tests based on gold particles coated with HIV antigens to detect HIV-antibodies in human blood (Auraflex). He was the first to succeed in making a specific gold label that worked in full serum. Moreover, he developed a new procedure to make spherical gold particles; this method was patented by Organon. In 1993 he worked a short period at CPRO-DLO and since March 1993 he works at the RIKILT in Wageningen. Starting at the Department of Microbiology, he developed a screening method for Yersinia enterocolitica in meat based on impedance measurement. In 1995 he started at the Department of Toxicology, introducing the DR-CALUX® and making it applicable for milk fat. Since then he was either involved in and/or responsible for the dioxin monitoring program. Together with his colleagues Ron Hoogenboom, Liza Portier and Karin van Ede, he experienced several dioxin incidents: the Belgian incident in 1999, the German bakery waste incident in 2003 and the koalinic clay (used to select potatoes for making French fries) incident in 2004.

From 1998 he is also responsible for the development of yeast-based assays for detection of hormonal activities in animal feed and urine. In 2002 the Rikilt yeast Estrogen bioAssay (REA) was developed and in 2004 he became a project leader at RIKILT in this field and validated the REA for calf urine and animal feed. In 2005 the Rikilt yeast Androgen bioAssay (RAA) was developed and used to identify the anabolic THG designer steroid in human urine.

Toine is married to Ingeborg Bovee-Oudenhoven, who he met at age 18. She also came to Wageningen to study Human Nutrition. Together they have two sons, Nick (21-3-1997) and Lars (17-5-2001), and one daughter called Esmee (28-10-1999). They were all born at their home in Wijchen.

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PATENT

In 1992 Organon Teknika bv patented a new gold sol procedure developed by T.F.H. Bovee.

CONGRESS PROCEEDINGS, SHORT ABSTRACTS, POSTERS, COURSES

During his career he followed several courses and attended several symposia at which he contributed with short abstracts and oral or poster presentations.

DANKWOORD

Het gereedkomen van een proefschrift is voor iedere promovendus een mijlpaal in zijn carrière. Hoewel nooit een “echte AIO” te zijn geweest, voelt het voor een (net) 40-jarige minstens zo bijzonder. Hoewel mijn toenemende grijze haren misschien anders doen vermoeden, kijk ik met heel veel plezier en voldoening terug op al die jaren labwerk, de vele discussies met collega’s (niet alleen) over resultaten, het brainstormen, het ontwikkelen en implementeren van nieuwe detectiemethoden, en het schrijven van nieuwe projectvoorstellen. Dit was niet mogelijk geweest zonder de samenwerking, hulp en input van velen, zoals……..

Allereerst natuurlijk Ron, jouw kennis en steun hebben een belangrijke bijdrage geleverd aan dit onderzoek. Gelukkig voor mij en vele anderen zijn jouw inzicht, rust en verbale vaardigheden van dien aard dat de boel nooit ontaard. Het respect dat je altijd voor anderen weet op te brengen en je terughoudendheid in discussies en optreden zorgen daarvoor. Je bent een heel goede schrijver van wetenschappelijke artikelen, ik heb veel van je geleerd en heb veel waardering voor het vele werk dat je (ook in je avonduren) voor het RIKILT verzet. Dankzij jouw inzicht en terughoudendheid is de CALUX uitgegroeid tot de beste screeningsmethode voor dioxinen en PCBs in tal van matrices. We hebben de afgelopen twaalf jaar al veel samen meegemaakt en met Astrid zijn wij drieën nu de langstzittenden in ons cluster. Ik hoop dat we samen nog vele jaren, op de voor jouw kenmerkende constructieve wijze, lekker en toegepast onderzoek kunnen blijven doen.

Michel, jouw komst op het RIKILT in 2002 was perfect getimed voor dit onderzoek. Was de analytische hormoonclub voor jou komst nogal gesloten, met jou hadden we al vrij snel intensief contact. Er waren al direct initiatieven om samen te werken en gezamenlijke projectvoorstellen in te dienen. En al was dit begin misschien nog enigszins sceptisch, voor en tijdens het MPA incident hebben we elkaar goed leren kennen. Na wat pilotexperimenten met pure urine, kwam jij met het voorstel om de REA te valideren voor urine van kalveren. Jouw kennis, enthousiasme en de voortvarendheid waarmee jij het onderzoek te lijf gaat, spreken mij enorm aan. Net als Ron ben je een dijk van een wetenschapper en schrijver. Ik hoop dan ook dat we nog lang zo kunnen doorgaan. Samen met Ron stonden we al eens in de krant als “de hormoonjagers van het RIKILT”. Ik ervaar het nog steeds als een soort drie musketiers gevoel.

Richard, als hardwerkende stagiaire begonnen op het RIKILT. Je kan heel veel verdragen van anderen en dat laatste heb je bij mij soms wel nodig! We hebben samen heel wat experimenten uitgevoerd. Dat was altijd heel gezellig en al waren de resultaten soms niet direct bevredigend: jij geeft nooit op. Jouw vasthoudendheid heeft er dan ook toe geleid, dat het RIKILT nu ook een van de beste bioassays voor de detectie van androgenen heeft: de RIKILT yeast Androgen bioAssay (RAA). Zoals je weet zijn we nog niet klaar en gaan we verder met de progesteron-achtigen en de corticosteroïden.

Liza, al ben je een beetje stil, jij bent sinds je komst op de afdeling (zo’n zes jaar geleden) wel RIKILT’s stille kracht op de CALUX. Ik kan niemand anders voorstellen die deze zware werkzaamheden gedurende al die jaren met zo’n precisie, gedrevenheid en kwaliteit had kunnen uitvoeren. We hebben vele jaren samen de monitoring voor onze rekening genomen en tijdens de dioxine-incidenten werd de hulp ingeroepen van onze naaste collega’s Gerrit, Richard, Astrid en Betty. Sinds eind 2004 is Karin erbij gekomen en bij het meest recente dioxine-incident in 2006 was ik al niet meer nodig. Jullie tweeën hebben die zaak keurig afgehandeld!

Astrid, de eerste RIKILT medewerker die bij Ron met de CALUX aan de slag ging. Toen ik erbij kwam, waren jullie al druk bezig met het testen van de specificiteit van deze assay. De uitslagen van de experimenten met de benzamidazolen en corticosteroïden waren vers. Het werd mijn taak om de assay toepasbaar te maken voor dioxines en PCBs in melkvet. Inmiddels heb ik je goed leren kennen. In je werk ben je zeer kritisch en al kan ik je soms niet volgen, als het kwartje bij mij valt, leidt het uiteindelijk wel altijd tot nuttig inzicht. Je bent onmisbaar in onze groep. Vaak vind je kleine klussen, die door de meeste van ons als hinderlijk worden ervaren, ontzettend leuk om te doen en ook van je creativiteit maken we graag gebruik!

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Henri, de switch van de chemische analyse naar de bioassays heb je moeiteloos gemaakt. Zonder jouw bijdrage hadden we nooit zo snel en zo’n goed validatie dossier in elkaar kunnen zetten. Gerrit en ik hebben veel van jou geleerd. Ik ben blij dat je nu naar ons cluster getransfereerd bent. Het kost wat, maar dan heb je ook wat.

De lijst van RIKILT medewerkers met wie ik veel en intensief heb samengewerkt is nog veel langer. Wim, Constant, Guido, Rob, Cornelis, Jaap, Liza en Karin bedankt voor de altijd prettige samenwerking. Dankzij jullie professionaliteit is de dioxine-monitoring een eitje en is de waardering van LNV en de VWA voor ons werk tijdens incidenten zo groot.

Jaap, Harry, Patrick en El, al was het soms lastig, op het moleculair biologisch lab heb ik een fantastische tijd gehad. Het was er altijd gezellig en dat was een hele goede voedingsbodem voor mij om hard aan gist te werken.

Paula en Johan, de fractionering voor het identificeren van de oestrogenen staat als een huis en ook met de androgenen lijken we weinig problemen te hebben. Nu nog even met Manja de boel valideren!

Ad en Hakan. Ad, je was een echte onderzoeker en nu ben je een echte clusterleider. Je pakt problemen direct aan, staat tussen de mensen en hebt het hart op de goede plaats zitten. Hakan, al werken we niet samen, voor mijn PC-problemen en het maken van tabellen en figuren, ben jij voor mij onmisbaar.

Robert en Adrie, met jullie als BUMmers, lopen de zaken op het RIKILT gesmeerd. Vergeleken met 12 jaar geleden, staat er nu een professionele organisatie. Vasthouden zo! Persoonlijk vind ik het jammer dat Kees nu bij het ATO zit.

Ivonne, ik ken je nog vanuit mijn studententijd en mijn afstudeervak van een half jaar op de vakgroep Biochemie. Jij was die intelligente en hardwerkende medewerker op de vakgroep die ooit wel eens Professor zou worden. Ik gaf het promoveren nooit echt een hoge prioriteit, maar jouw komst als hoogleraar Toxicologie gaf mij het zetje dat ik nog nodig had. Ondanks je drukke werkzaamheden was je altijd op korte termijn bereikbaar voor discussie en het beoordelen van manuscripten.

Pap en mam, bedankt voor alle steun door de jaren heen. Als er niets meer is, dan zijn jullie er altijd nog.

Piet en Elly, de dinsdag is altijd een feest voor de kinderen en ook voor ons. Het woord schoonouders, en alle gangbare associaties die dat oproept, is totaal niet van toepassing op jullie!

Ing, bedankt voor je liefde en steun. Jij zet de dingen voor mij vaak in het juiste perspectief. Nick, Esmee en Lars, wij zijn zo trots op jullie en niet alleen omdat we alle ‘’goedjes’’ op jullie rapporten amper kunnen tellen. Mijn screensaver op het RIKILT moet hoognodig worden uitgebreid met een paar mooie foto’s van jullie.

Cover design: Hakan Baykus

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