Development of a planar yeast estrogen screen as screening tool for estrogen active compounds

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Abbreviations

6-PN	6-prenylnaringenin
8-PN	8-prenylnaringenin
AP	alkylphenol
APEO	alkylphenol polyethoxylates
BPA	bisphenol A
CPRG	chlorophenol red β-D-galactopyranoside
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
e.g.	exempli gratia
E1	estrone
E2	17β-estradiol
E3	estriol
EAC	estrogen active compound
ED_{50}	half maximal effect dose
EDC	endocrine disrupting chemical
EE2	17α-ethinylestradiol
EEA	estradiol equivalent amount
EEF	estradiol equivalent factor
EEQ	estradiol equivalent concentration
EQS	environmental quality standards
ER	estrogen receptor
ER-CALUX	ER-mediated chemically activated luciferase gene expression assay
ERE	estrogen-responsive element
et al.	*et alii*
EU	European Union
EV_{50}	half maximal effect volume
hER	human estrogen receptor
HPTLC	high-performance thin-layer chromatography

HPTLC-pYES	high-performance thin-layer chromatography-planar yeast estrogen screen
i.e.	*id est*
IPCS	International Programme on Chemical Safety
IXN	isoxanthohumol
lacZ	gene of the *lac* operon in *Escherichia coli*
LC	liquid chromatography
LLE	liquid-liquid extraction
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MU	methylumbelliferone
MUG	4-methylumbelliferyl-β-D-galactopyranoside
NP	nonylphenol
NPE	nonylphenol ethoxylates
OP	octylphenol
pYES	planar yeast estrogen screen
RGP	resorufin-β-D-galactopyranoside
RNA	ribonucleic acid
RP-18 W	water-wettable reversed phase
RSD	relative standard deviation
SPE	solid phase extraction
USA	United States of America
UV	ultraviolet
WHO	World Health Organization
XN	xanthohumol
YES	yeast estrogen screen

Preliminary remarks

The research work presented in this thesis was carried out under the supervision of Prof. Dr. Wolfgang Schwack at the Institute of Food Chemistry, University of Hohenheim, Stuttgart, Germany, between August 2013 and September 2017.

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Full publications

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Contributions

The participation and contributions of the authors to the specified full publications are as follows:

Dinah Schick performed all essential practical and analytical work. Analysis and interpretation of the obtained data was carried out by her, including the preparation of the original manuscripts that led to the publications.

Prof. Dr. Wolfgang Schwack was the supervisor of the work presented in this doctoral thesis. The idea for the development of a modified pYES and the proposal for an alternative evaluation were initiated by him. He advised in analytical issues and proofread the original manuscripts that led to the specified publications, and he was responsible for all formal aspects of the publication processes.

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I General introduction

1 The endocrine system and endocrine disruption

The regulation of various processes and the communication in the body by means of the interaction of glands, hormones and receptors is referred to as the endocrine – or hormone – system. The synthesis and secretion of hormones is carried out by distinct glands and cells that are located at different sites of the body [1], while several hormones can be synthesized by the same gland [2]. Hormone-sensitive parts of the human body are demonstrated in the illustration below (Figure 1).

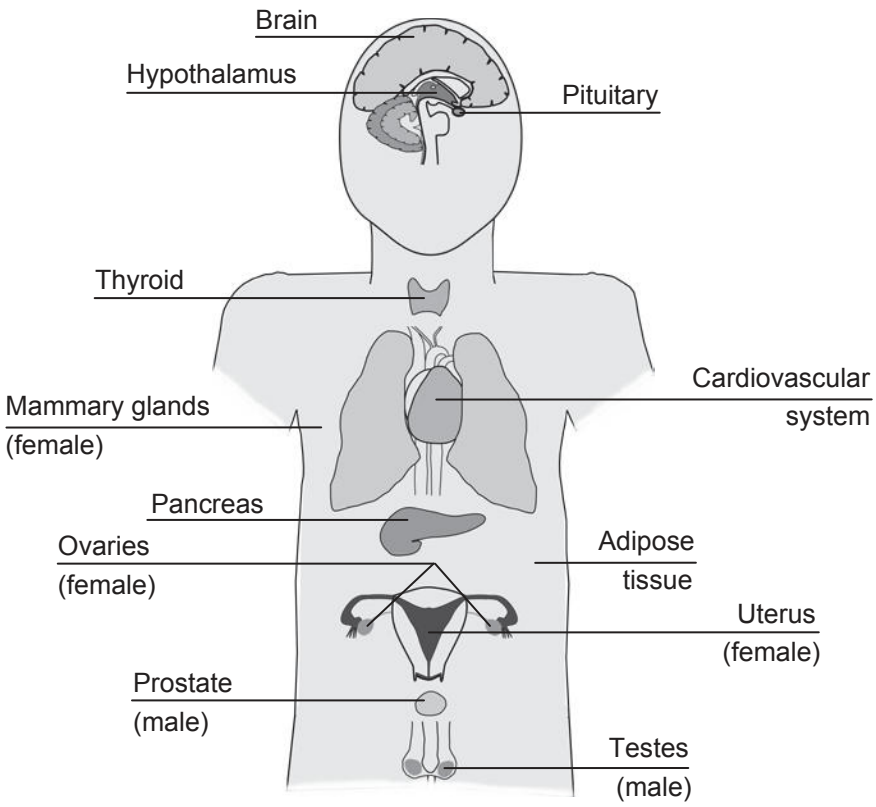

Figure 1 Outline of the endocrine system showing the hormone-sensitive sites of the human body (model according to [3])

Hormones are essential for the communication and other functions in the body and are important signaling molecules that are transported through the bloodstream until they reach their target tissues or cells [1,2]. In these tissues and cells, the hormones are bound by respective receptors causing distinct effects or responses such as the production of specific substances or specific changes in the metabolism [1,2]. At this, the interaction of hormones and receptors is dependent on both the type of the hormone and the receptor. Derived from different precursors (e.g. cholesterol is the precursor of steroids) the four classes of hormones are i) steroids, ii) amines leading to thyroid hormones and catecholamines,

iii) peptides/proteins, and iv) eicosanoids [1]. The major receptor classes are receptors on the cell surface (membrane receptors), in the cell cytoplasm and in the cell nuclei (both intracellular receptors), binding different types of hormones [1].

Distinct biological and physiological processes in the human body are controlled by different hormones. For example, estrogen and testosterone are involved in the development and function of the reproductive system, insulin in the control of the blood sugar, further hormones like thyroid hormones are important for other regulations such as the development of the brain and the nervous system [1]. Therefore, hormones and the entire endocrine system play an essential role for human health while any disruption can have serious consequences like disease or death [2]. At this, a disruption can have 'natural reasons' (medical condition like genetic defects) or can be caused by exogenous influences (ingested, inhaled or absorbed substances). Possible consequences might be problems during growth, disturbances of metabolic processes or the brain function due to impaired thyroid hormones but also infertility or other reproductive problems when the normal balance of sex hormones is disrupted [2]. Even death can be the consequence of a disrupted hormone system, like in patients with a type I diabetes if their lack of insulin is not treated [2].

Any interference of the endocrine system or any influence on it by diverse chemicals or substances called endocrine disrupting chemicals (EDC) is referred to as endocrine disruption. The World Health Organization (WHO) stated the following definition of a (potential) endocrine disruptor in the course of the International Programme on Chemical Safety (IPCS 2002): "*An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.*" [4–6].

The disruption of the endocrine system by EDC is effected *via* various mechanisms, mainly *via* steroid hormone receptors that are part of the nuclear receptor superfamily and that regulate transcriptional activation [7]. Besides, also other mechanisms are involved, for example, mechanisms *via* nonnuclear steroid hormone receptors like membrane receptors, *via* non-steroid receptors and others [3].

Intracellular receptors are activated by binding ligands resulting in conformation changes, and subsequently the activated receptors bind to responsive elements of genes to initiate their transcription [8]. Consequently, messenger ribonucleic acid (mRNA) is produced by means of the enzyme RNA polymerase, which conveys the information from the cell nucleus into the cytoplasm where it is translated at ribosomes resulting in the production of the target

protein [8]. At any time, these steps can be interfered, while an EDC can not only act as a hormone mimic, but can also be an antagonist or inhibitor, influencing the normal physiological processes of the body. An EDC, thus, can have impacts on metabolic processes such as the synthesis, excretion and metabolism of hormones, can alter hormone receptor levels or can cause disorders in the transport of hormones but also can interfere associated translations and transcriptions [1,5,9,10]. Furthermore, an endocrine disruption can cause alterations of signaling pathways, development and other biological processes [1].

A number of different scientific institutions work in this field of research and a large number of literature is concerned with this often reviewed topic (see reviews [3,9,11]). Amongst other things, EDC are blamed for influencing various stages of the development of organisms, and for having negative effects on the fecundity as well as for being responsible for birth defects and metabolic disorders in mammals, fish and birds, as well as in reptiles and mollusks in Europe, North America and other localities [12,13]. For many of known or suspected EDC that occur in our environment the actual effects on humans and wildlife are, however, still uncertain, while for some EDC the connection between the specific substance and its disrupting properties was confirmed.

Amongst the great spectrum of compounds with biological activity at the endocrine system *inter alia* natural and synthetic hormones, organotins [14], organochlorines [15], alkylphenolic compounds [16,17], and cosmetic UV filters [18,19] are involved, but also other chemicals and substance groups have been proved to be active at the endocrine system or are supposed to be EDC. A great number of these substances are persistent, lipophilic and have a low vapor pressure, why they – supported by the global transport – are to be found all over the world [20]. Others, however, have a shorter lifespan and can be detected in higher amounts near their sources [6]. Additionally, EDC also may be formed by *in vivo* activation or (bio)degradation resulting in endocrine active (breakdown) products [5,6,20]. Because of the various characteristics and sources, EDC occur in soil and air as well as in water (groundwater, surface water) [5,6]. Furthermore, EDC are substances that are used very commonly, e.g. during the production of plastics, commodities or in formulations of plant protection agents or cleaning products, and even can accumulate in the food chain, so as to an exposure with EDC *via* a number of routes is possible. This comprises drinking, breathing, diet, but also exposure through direct contact, e.g. at work or *via* consumer products [3]. Moreover, for infants and fetuses, a possible exposure through their mothers is of great importance and concern [2].

In the 1990s, studies presented an observed reduction of the sperm count and seminal volume in men and simultaneously an increasing number of abnormalities in the genitourinary system like cryptorchidism, testicular cancer and hypospadias over the period of the preceding 50 years [21,22]. This was assumed to be related to environmental influences, *inter alia*, due to a simultaneously occurring increase of EDC in the environment, since genetically changes are unlikely in this rather short period of time [21]. Furthermore, it was also assumed that an increasing exposure with estrogen active compounds (EAC) lead to disorders of the male reproductive tract [23], especially when exposure took place *in utero* since, for example, testicular cancer is presumed to be initialized early in life [24]. Henderson *et al.* suspected exogenous hormone use during pregnancy, particularly during the time of differentiation of the testes of male fetuses, as a major risk factor for testicular cancer in young men [25]. Moreover, a study in Denmark concluded that elevated estrogen levels during first pregnancies pose a risk for testicular cancer in male offspring (first born sons) [24].

Such studies and observations demonstrate that not only exposure to endogenous or exogenous natural hormones but also exposure to other endocrine active substances occurring in our environment can lead to a disruption or to alterations in humans and wildlife. This is also true for endo- and exogenous natural estrogens and EAC, which is to be dealt with in the ensuing chapter.

2 Estrogens and estrogen active compounds (EAC)

2.1 Assays for the detection of estrogens and estrogen active compounds

Several *in vivo* and *in vitro* assays were developed for the detection and determination of estrogen active compounds (EAC). According to Leusch, the application of bioassays is of advantage compared to other chemical (chromatographic) methods since the specific chemical nature of the substances does not have to be known and lower detection limits are achievable [26]. By using most of the available bioassays, admittedly the estrogenic activity of a sample or of specific substances can be detected, but the identity of the individual estrogen active components of a sample cannot be determined. In order to detect and determine single compounds, therefore, usually an additional analysis, for example with high-performance liquid chromatography or gas chromatography, is required. In case of unknown substances, an identification employing mass spectrometry (MS) can be performed.

The development of the planar yeast estrogen screen (pYES), however, provided the option to separate the single EAC of a sample by high-performance thin-layer chromatography (HPTLC), and subsequently detect and determine them in the same assay. At this, the compounds of a sample are chromatographically separated on HPTLC plates, thus in a planar system, and the detection of the different EAC with genetically modified yeast cells is performed directly on the plate. Employing pYES, therefore, provides the information of interest of a whole sample all at once. Additionally, the possibility for an identification of unknowns is realized by directly coupling HPTLC to MS.

Without regard to the commonly used standard chromatographic methods, certain *in vivo* and *in vitro* assays for the detection of EAC, including the planar yeast estrogen screen (pYES), are presented hereafter.

2.1.1 *In vivo* assays

In vivo methods provide information about the effects of compounds in organisms or the impact on their metabolism. For the detection of endocrine disruptors and EAC, a wide range of *in vivo* methods were developed and two of these methods – the rodent uterotrophic assay and the pubertal female rat assay – will be exemplarily explained.

The rodent uterotrophic assay is a method that is based on the uterotrophic response to estrogens. Present EAC cause a rapid growth of the uterus in immature or ovariectomized rats normally showing constant and low uterine weights [27]. The rats are treated – usually by oral gavage – with the test substances for three days, and 24 h after the last dose, the

uterus weight is determined and compared to reference values [27]. The test shows good reproducibility, but only slight uterine weight increases can lead to false positive results [27].

Another test is the pubertal female rat assay that is based on the detection of different changes in the organism caused by the test substance administered [28]. Pubertal female rats (22 days old, weaned at day 21) are treated for 20 days with the test substance and different endpoints are recorded, e.g. body weight, thyroid histology, uterine and ovarian weights and histology, vaginal cytology, and others [28]. The test thus provides a statement of the effects of the test substance on the pubertal development and thyroid function of the rat [28].

2.1.2 *In vitro* and cell-based assays

In contrast to *in vivo* assays, *in vitro* and cell-based methods do not consider the entire organism and effects on it, but certain substeps of functional chains, e.g. the binding of a ligand by estrogen receptors (ER) [29]. Examples for *in vitro* assays for the detection of estrogenic activity are the ER-mediated chemically activated luciferase gene expression assay (ER-CALUX) [30], the E-Screen (proliferation assay with MCF-7 breast cancer cells) [10] and the yeast estrogen screen (YES assay) [17].

In the course of the development of the ER-CALUX, luciferase reporter genes were implemented in different cell lines [30]. The implementation of the reporter gene in T47D human breast cancer cells expressing the ER provided highly responsive cell lines for the detection of (anti-)estrogenic activity of the test substances [30]. The test is based on the binding of present EAC by the ER, which is thus activated and subsequently binds to an estrogen-responsive element (ERE), resulting in transcriptions that lead to luciferase expression [30]. After addition of luciferin, the enzyme activity, and thus indirectly the estrogenic activity, is measured in a scintillation counter [30].

The E-Screen, in contrary, uses MCF-7 human breast cancer cells and investigates cell proliferation after exposure with the test substances or samples [10]. In presence of EAC, the proliferation of the MCF-7 cells is induced and an increase of the number of cells compared to a negative control (absence of EAC) is observed, *i.e.*, the proliferation behavior is determined by means of measuring the cell yield [10].

Amongst available *in vitro* tests for estrogenic activity, particularly the yeast estrogen screen (YES assay) is to be mentioned. The assay is performed with yeast cells of a recombinant strain of *Saccharomyces cerevisiae* [17]. The yeasts are modified with a DNA sequence of the human estrogen receptor (hER) and the *lacZ* reporter gene that encodes for the enzyme

β-galactosidase [17]. By binding of EAC, the hER is activated and subsequently binds to an ERE causing gene transcriptions that lead to the expression of the reporter gene and thus to the production of β-galactosidase [17]. The glycosidic bond of the substrate chlorophenol red β-D-galactopyranoside (CPRG) is cleaved by the enzyme and releases chlorophenol red, which subsequently is determined by absorbance, *i.e.*, the estrogenic activity is measured indirectly *via* the enzymatic cleavage of a suitable substrate [17]. The YES assay is executed in aqueous solutions in microtiter plates, delivers results in 3–4 days, and provides a statement about the total bioactivity of the samples (for example as equivalents of 17β-estradiol), but neither the individual EAC nor their identities are determined. Since not only estrogenic but also anti-estrogenic effects or even toxicological effects of the entire sample or sample extract are involved in the outcome of the assay, the result is obtained as total estrogenicity or rather as the sum of the biological activity of the components of a sample.

2.1.3 Planar yeast estrogen screen (pYES)

The transfer of the YES assay (see chapter 2.1.2) to a planar system was introduced by Müller *et al.* employing HPTLC plates silica gel 60 F_{254}, and CPRG and 4-methylumbelliferyl-β-D-galactopyranoside (MUG), respectively, as substrates [31]. The HPTLC-YES (planar YES, pYES) provides the option to determine EAC directly on the HPTLC plate. For that purpose, the test substances were applied onto the plate which was immersed in a yeast suspension of genetically modified yeast cells according to Routledge and Sumpter [17] afterwards and subsequently was incubated [31]. Like in the microtiter plate YES assay described above, β-galactosidase is produced in consequence of the binding of EAC by the integrated hER and estrogenic activity was measured indirectly by means of the signal of the cleaved substrates in the respective HPTLC zones. The use of the fluorogenic substrate MUG, releasing blue fluorescent methylumbelliferone (MU) after enzymatic cleavage, increased the sensitivity of the screen by a factor of 20 compared to the chromogenic substrate CPRG releasing chlorophenol red [31]. Employing an HPTLC method usually provides the advantage to separate analytes due to their different properties in a planar chromatographic system and to see the single compounds of a sample after chromatography at one look. However, no separation of analytes by chromatography was performed in the work of Müller *et al.*, and the duration of the test was at least 29 h [31].

Almost 10 years later, the planar YES as a combination of HPTLC and effect directed analysis for EAC on HPTLC silica gel plates with MUG as substrate was presented by Buchinger *et al.* [32], Spira *et al.* [33] (both worked with yeast cells generated by McDonnell

et al. [34,35], see below) and Schönborn and Grimmer [36] (yeast cells according to Routledge and Sumpter [17]). The mentioned studies demonstrated the separation and determination of EAC by pYES after ≤5 h. However, due to the aqueous conditions during pYES employing silica gel plates, the tests resulted in broadened and relatively diffuse HPTLC zones, complicating a quantitation. In 2017, Schönborn *et al.* presented a modified method for yeast cell application by a spray-on technique of cell suspensions onto the silica gel HPTLC plates instead of an immersion of the plates into the suspension, while, however, also the cell density was increased fivefold [37]. The spraying-on of the yeast suspension (yeast cells according to McDonnell *et al.* [34,35], see below) obviously led to sharper HPTLC zones and additionally lower limits of quantitation for the tested compounds 17β-estradiol (E2), estrone (E1), 17α-ethinylestradiol (EE2) and estriol (E3) compared to former results and results presented for water-wettable reversed phase plates (HPTLC RP-18 W plates, see below), respectively.

In 2014, however, Klingelhöfer and Morlock already modified the screening by using HPTLC RP-18 W plates, resulting in sharp-bounded, blue fluorescing zones of MU [38], using yeast cells according to McDonnell *et al.* [34,35]. McDonnell *et al.* generated a recombinant yeast strain of *Saccharomyces cerevisiae* by constructing an expression vector for the hER and integrating it in the yeasts [34]. Moreover, the yeast cells contain a so-called *lacZ*-CYC fusion reporter plasmid, composed of the yeast *iso*-1-cytochrome c proximal promoter elements and the structural gene (*LacZ*) for β-galactosidase from *Escherichia coli* [34,39,40]. In the course of chromatography, the components of a sample are separated on the HPTLC plate and are to be found in different HPTLC zones afterwards. EAC are bound by the implemented hER, causing the expression of the reporter gene and, therefore, the production of the enzyme β-galactosidase, resulting in the enzymatic cleavage of the substrate MUG. Estrogenic activity is then indicated through blue fluorescence of released MU in the HPTLC zones of present EAC.

During their experiments, Klingelhöfer and Morlock figured out the requirement of a pH adjustment of the RP-18 W plates since the plate material showed a low pH value of 4.8 [38], and the activities of both yeast cells and enzyme are *inter alia* dependent on the current pH. For yeast cell activity, the pH of 4.8 is suitable since optimal metabolism and growth of yeasts are reported between pH 4.5 and 6.5 [41]. The optimal pH for the maximum activity of β-galactosidase, however, was reported at about 7.2 with enzyme stability between 6 and 8 (*E. coli*, substrate o-nitrophenyl-β-D-galactoside) [42]. Therefore, Klingelhöfer and Morlock used a citrate buffer with a pH of 12 for the preparation of the MUG solution, resulting in a pH

of 7.2–7.4 on the plate surface after immersion in the substrate solution [38], which matched the β-galactosidase pH optimum. Moreover, an additional stop solution for the termination of the substrate reaction with the same pH was applied [38]. MU has, however, a relatively high pK_a of 7.8 [43] and would be present in its high fluorescent anionic form with only about 30% at a pH of 7.4. Since the fluorescence signals of MU obtained in the work of Klingelhöfer and Morlock are very intensive, however, the final pH of the plate is assumed being greater than 7.4, presumably due to the high pH of the stop solution.

The application of the pYES developed by Klingelhöfer and Morlock with the reversed phase, water-wettable HPTLC plates on samples like beer, hops, waste water, river samples, propolis and spices, and the coupling to MS in order to identify unknown EAC was demonstrated [38,44–46]. The diverse samples were analyzed after extraction mostly without further purification steps and it has become apparent that many samples originally contain contaminants showing native fluorescences (blue, red) [45,46]. Blue fluorescing zones of sample components, however, might interfere with the determination of the estrogenic analytes when using the substrate MUG, since blue fluorescing MU is released as positive signal for estrogenic activity. To prevent false-positive conclusions, consequently negative controls had to be executed by performing the test without yeast cells [45,46]. By comparing negative controls and pYES results after substrate incubation with MUG, the differentiation of blue fluorescing zones due to native fluorescence and blue fluorescing zones due to estrogenic activity (enzymatically released MU) was possible [45,46]. Alternatively, the use of another substrate was suggested, e.g. the chromogenic substrate CPRG [46], releasing chlorophenol red after enzymatic cleavage.

Compared with the YES assay performed in microtiter plates, the pYES provides the detection and determination of single, separated EAC directly on the HPTLC plate, which is of high advantage. Consequently, anti-estrogenic and toxicological effects of other substances do most likely not disturb the determination of the substances of interest, since the single components occurring in the sample are separated from each other, and the EAC can thus be detected in single HPTLC zones.

New developments in research in terms of the detection of endocrine disruptors with biosensors were recently presented by Chamas *et al.* (2017) [47,48]. Three modified yeast strains of *Arxula adeninivorans* were introduced, producing recombinant human estrogen, androgen and progestogen receptors, respectively, and additionally different reporter proteins showing differing fluorescences (blue, green, red) in response to the binding of

respective ligands [47]. The simultaneous detection of estrogens, androgens and progestogens was successfully shown in microtiter plates after mixing the strains followed by the detection of the produced fluorescences at distinct wavelengths [47]. Additionally, the newly developed reporter assay was transferred to TLC (silica gel plates without fluorescence markers), allowing the separation and detection of the hormone activity of single substances of a sample [48]. The new three-channel bioassay, thus, provided the detection of the different sex hormones, while an additional step of substrate incubation (production of an enzyme which cleaves a suitable substrate resulting in a measurable signal as evidence for hormone activity) was no longer required [47,48].

2.2 Estrogen active compounds

All the stated aspects and adverse effects of endocrine disrupting chemicals (EDC, chapter 1) also hold true for EAC. These compounds can be natural or synthetic estrogens as well as xenoestrogens (natural or synthetic chemical substances with an estrogenic effect that occur in the environment or are used in consumer goods and other products). At natural estrogens, it is to be dealt with human estrogens or compounds like phytoestrogens, e.g. from soy [49–51] or hops [52]. Synthetic estrogens are substances used in pharmaceuticals, such as the estradiol receptor agonist 17α-ethinylestradiol that is used in contraceptives. Xenoestrogens are, for example, mycotoxins like the *Fusarium* toxin zearalenone [53], organochlorine pesticides like dichlorodiphenyltrichloroethane [54] or UV filter substances used in cosmetics [18,19].

The principal targets of estrogens and EAC are the estrogen nuclear receptors ERα und ERβ [7]. Both receptors usually are located in different tissues but also can occur in the same tissue but in different cell types [55]. Mainly, ERα occurs in the ovary (theca cells), uterus, bone, breast, testes but also in the prostate (stroma), brain and white adipose tissue, while ERβ generally is to be found in the epithelium of the prostate, the granulosa cells of the ovary, testis, bone marrow, colon and also in the brain [55]. In ER-containing tissues of the body, EAC can be bound by the ER resulting in activation. The activated ER binds to ERE of the DNA, causing the transcription and therefore the production of target proteins, when mRNA is translated. Thus, EAC can impact various cellular processes in different tissues and sites of the endocrine system by binding to one of the receptors resulting in stimulation or inhibition of estrogen-responsive genes, which might lead to changes in the expression of other genes [7]. The effect of EAC is not only dependent on the receptors themselves, but also on the type of the ligand or different signaling pathways [7]. Therefore, the same ligand can have various effects by binding to different ER in distinct tissues [55], like the non-steroidal anti-estrogen tamoxifen that is used in hormone-dependent breast cancer therapy. Tamoxifen acts as anti-estrogen in breast cells, while it showed estrogenic effects in experiments in old ovariectomized rats, when it stabilized body weight and reduced the loss of bone, albeit ovariectomized rats normally show osteoporotic symptoms due to the deficiency of estrogens [56].

Regarding the aquatic environment, some studies suggested that there is only a negligible risk of EAC exposure to humans through waters. However, EAC can accumulate in wildlife species, as presented in [57]. In the stated study, fish near a waste water treatment plant were analyzed, and showed increased levels of 17α-ethinylestradiol in the bile of fish

downstream compared to fish upstream the plant [57]. Larsson *et al.* therefore supposed EAC to be a health risk for humans due to their accumulation in fish and other predatory animals [57]. Additionally, it has been shown that EAC can have additive effects even if the single components are present in concentrations less than their no observable effect concentrations [58]. Similar observations in the course of their experiments were made by Sumpter and Jobling, when a mixture of weakly EAC resulted in a higher response than the sum of the responses of the single components [59].

Since estrogens and therefore also substances with estrogenic activity play a decisive role in human health, physiology and also in various diseases [55], the wide range of EAC with different structures and sources is of high concern. Estrogens are *inter alia* implicated in breast tumors or disorders of the brain, but also in changes in the development, bones and the cardiovascular system [55]. The large variety, ubiquitous presence and additive effects of EAC, and their impact on wildlife and humans is known and partly proved but cannot be estimated on the whole, and only a couple of examples of the following EAC classes i) human estrogens, ii) synthetic estrogens, iii) phytoestrogens and iv) xenoestrogens, and their effects, will be thematized hereafter.

2.2.1 Human estrogens

The hormones 17β-estradiol (E2), estrone (E1) and estriol (E3) are human sex steroids and the most important natural estrogens. The chemical structures of E2, E1 and E3 are very similar and differ merely in their substituents at the sterane skeleton (Figure 2).

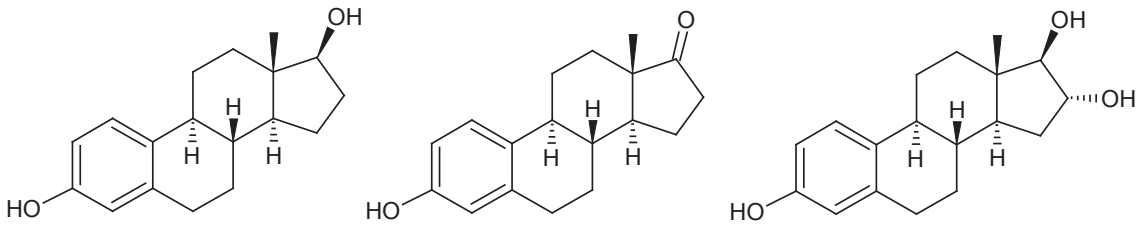

Figure 2 Chemical structures of the natural human estrogens 17β-estradiol (E2), estrone (E1) and estriol (E3)

The biosynthesis of human estrogens from cholesterol is a tissue specific process, *i.e.*, the different estrogens are synthesized at different sites of the human body [8]. E2 is synthesized in the ovary, whereas the synthesis of E1 takes place in the adipose tissue, and the synthesis of E3 in the placenta [8]. In non-pregnant, fertile women, E2 is the most important estrogen and additionally is the most potent of the endogenous estrogens [60]. In contrast,

E3 is mainly produced during pregnancy, whereas E1 is in metabolic equilibrium with E2 and moreover the predominant estrogen in postmenopausal women, when E2 synthesis decreases [60]. Estrogens are essential for various developments and mechanisms in both female and male [8]. They regulate important processes in the human body and influence, for example, the development and function of the female reproductive tract, the sperm transport, the endometrial development and tubal transport mechanisms [8]. Other target tissues of estrogens are the liver, the bones, the central nervous system, the cardiovascular system, and the skin [60].

When respective hormones are produced in insufficient amounts, disorders or afflictions can occur. Such a deprivation can thus be treated by hormone administration. The medical use of hormones is a long known and used therapy for different diseases or complaints. A common therapy, for example, is the treatment with estrogens and progestogens (gestagens, which are also steroidal sex hormones) to decrease (post)menopausal complaints in elderly women, when the amount of endogenous hormones becomes insufficient [61].

2.2.2 Synthetic estrogens

Synthetic estrogens are substances that were developed and synthesized with the aim of being estrogen active and that are used as active ingredients in contraceptives and other pharmaceuticals. Examples for synthetic estrogens are the steroidal substances 17α-ethinylestradiol (EE2) and mestranol (a precursor of EE2), or the non-steroidal diethylstilbestrol (DES).

Like the natural estrogen E2, the synthetic estrogen EE2 shows activity at estrogen receptors and is therefore used in contraceptives as ER agonist. Structurally, EE2 is similar to E2, and likewise shows the sterane skeleton such as the natural hormone, however, also shows an additional ethinyl group as substituent at C-17 (Figure 3).

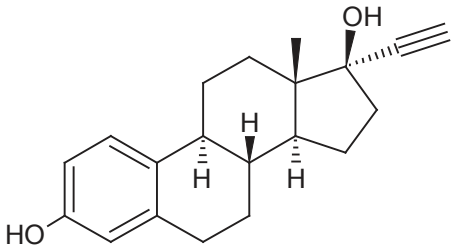

Figure 3 Chemical structure of 17α-ethinylestradiol (EE2)

EE2 was developed to be active as ER agonist in humans for contraception, but it has been observed to also have an impact on wildlife species. After the finding of hermaphrodite fish in lagoons of waste water treatment plants, it was assumed that sewage and substances like EE2 occurring in the waste water caused the physiological disturbances in local fish, why tests for vitellogenin production in fish were executed [62]. Vitellogenin is a precursor protein normally produced in maturing female fish induced by estrogens and is used as biomarker for EAC [59]. The possibility for an evidence of estrogenic contamination *via* the vitellogenin production is given since the content of vitellogenin in male fish is very low or vitellogenin is not present at all [63]. However, vitellogenin production in male fish can be induced by EAC [59]. Therefore, rainbow trout were exposed to EE2 by injection or immersion in EE2-containing water, and vitellogenesis was investigated [62]. The experiments showed the induction of vitellogenin production and thus estrogenic activity of EE2 even at low concentrations of 0.1 ng/L in the test water [62]. Therefore, EE2 was proven to be a very potent EAC in fish [62].

A further study investigated the effects of EE2 (mean of ~6 ng/L) on wild fathead minnows in test lakes compared to fish from reference lakes (no addition of EE2) over several years, when vitellogenin production in male and also an increased vitellogenesis in female fish were observed [64]. The production of vitellogenin in female fish collected in the non-active vitellogenic stage in fall did, moreover, not really decrease whereas vitellogenesis in female fish from the reference lakes declined [64]. Changes in liver cells and abnormalities in the kidneys in both male and female fish were also observed [64]. Moreover, male fish showed fibrosis and feminization, and the development of the testicular tissue was interfered [64,65]. In addition, abnormalities like a modified oogenesis were also observable in female fish [64,65]. All the stated aspects led to a population collapse, whereby *inter alia* also the short life cycle of fathead minnows was supposed to be a relevant factor [64,65].

Studies on sand gobies in Scotland also showed the influence of EE2 on a fish population, when sand gobies were exposed in a flow-through experiment to water containing EE2 (6 ng/L), resulting in reduced reproductive success and elevated vitellogenin levels [66]. Similar experiments were executed in Finland, when male sand gobies were exposed to EE2 (5 and 24 ng/L) and the effects on the sexual selection and the mating system but also physiological effects *via* the biomarkers vitellogenin and zona radiate protein (an eggshell protein) were investigated [67]. The studies showed a disrupted sexual selection most likely caused by the behavior of male fish and it was assumed that an increased period of exposure would lead to the loss or reduction of secondary sexual characters and therefore to reproduction problems for the population [67]. The studies also showed effects on the behavior of male fish at low concentrations of EE2 (5 ng/L) at which no production of

vitellogenin or zona radiate protein was induced, why the authors pointed out that these biomarkers should not be the only monitored parameters at investigations of the reproductive behavior [67].

A further synthetic chemical produced for contraceptives is mestranol, which, however, is inactive as estrogen but is a precursor of estrogen active EE2 and also shows structural similarity to E2 (Figure 4).

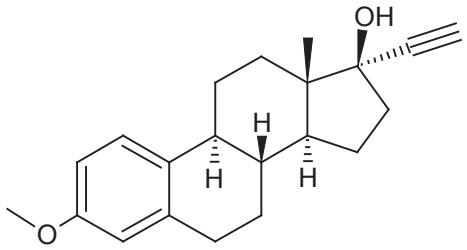

Figure 4 Chemical structure of mestranol

Mestranol was used in combined compounds for contraception. As prodrug of EE2, mestranol has to be transformed into the estrogen active form of EE2 *in vivo* by demethylation in the liver [68]. Due to variable conversion in the body, however, the actual amount of active EE2 cannot be estimated, why mestranol usually is not used in contraceptives anymore [68].

Although the compound diethylstilbestrol (DES, Figure 5) does not show clear structural similarities to E2 or other natural steroidal estrogens, the substance shows estrogenic activity.

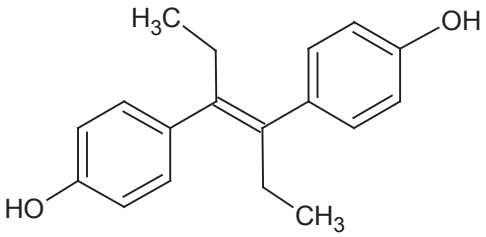

Figure 5 Chemical structure of diethylstilbestrol (DES)

DES is a non-steroidal synthetic estrogen that was developed in the 1930s, and was used back in the 1940s until the beginning of the 1970s in pregnant women to prevent spontaneous abortions and other pregnancy complications [20]. In the early 1970s, however, studies revealed that about 60% of *in utero* to DES exposed women, thus daughters of DES-

treated pregnant women, showed structural changes or abnormalities in their reproductive organs in adulthood [69]. Additionally, DES did not only impact *in utero* exposed female offspring but also *in utero* exposed sons. It has been observed that men exposed to DES *in utero* showed crucially more often genital malformations than non-exposed men [70]. These observations were made twice as often in male offspring that were exposed to DES in the first 11 weeks of pregnancy compared to men exposed later in gestation [70]. Therefore, a higher risk for a diagnosis of genital malformations in men, who were exposed to DES during early pregnancy, was assumed [70]. Other effects such as infertility of the exposed sons were not observed [70].

2.2.3 Phytoestrogens

Alongside endogenous estrogens or pharmaceutical treatments, an exposure to EAC can occur through further routes. Other natural sources for EAC are plants containing phytoestrogens, when an exposure can take place, for example, through diet or by direct contact. As to be seen hereafter, the discussed phytoestrogens also do not show clear structural similarities to the natural human hormones. Nevertheless, their estrogenic activity is proven.

It was reported, for example, that women, who harvested hops by hand, suffered from disturbances in their menstrual cycle [71]. At first, the prenylchalcone xanthohumol (XN), the most abundant hop flavonoid [72], was assumed to be responsible for the estrogenic properties of hops [71]. Later studies, however, revealed 8-prenylnaringenin (8-PN, Figure 6) to be a potent phytoestrogen and the hop flavanones 6-prenylnaringenin (6-PN, Figure 6), 6,8-diprenylnaringenin and 8-geranylnaringenin to be further hop compounds showing estrogenic activity [52,73].

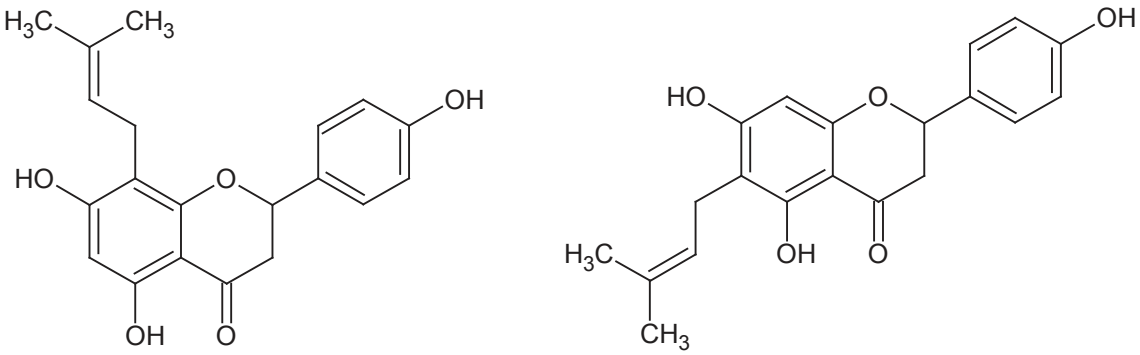

Figure 6 Chemical structures of the prenylated hop flavanones 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN)

During the brewing process of beer, the major hop flavonoid XN can be transformed to isoxanthohumol (IXN), and also the formation of the estrogen active prenylated hop flavanones 6-PN and 8-PN from the isobaric estrogen inactive desmethylxanthohumol was reported [74,75], why more prenylflavanones than prenylchalcones are present in beer [76]. IXN was determined as major prenylflavonoid in beer [76] and represents a so-called proestrogen, *i.e.*, IXN can be converted into 8-PN by specific intestinal microbiota in humans, which leads to a probably higher exposure to EAC than be expected after an analysis of beer samples [77,78].

Further phytoestrogens can be found in various plant species, for example in some strains of clover [79] or in soy beans [49,50]. Clover species contain isoflavones such as genistein and biochanin A, but also formononetin that can intestinally be transformed into the estrogenic active isoflavan equol [79]. The estrogenic activity of compounds from strains of subterranean clover was detected at the end of the 1940s, when sheep (ewes) grazing on clover-rich areas in Western Australia suffered from infertility due to estrogenic clover isoflavones [79]. Shortly after these observations, experiments with ovariectomized sheep were executed [80]. Ovariectomized sheep usually show an atrophy of the uterus, which can be treated by estrogen administration therapies, *i.e.*, uterus atrophy can be restored by treatment with estrogens [80]. To consider whether a clover-rich diet will have equal effects on ewes, ovariectomized sheep were divided in two groups, one of which was held on an area dominated by clover, and the other group on a control pasture without clover, and the sheep were examined in certain intervals [80]. During periods of growing green clover, the animals of the group on the clover-rich field did not show effects upon ovariectomy, while the animals of the control group showed changes of the uterus [80]. In times when clover wilted, however, an atrophy of the uterus was observable in both groups [80]. The possible restoration of the normal uterus state after atrophy by grazing on green clover pastures in the following season was also observed [80]. These experiments thus proved the estrogenic potential of clover.

Exposure to phytoestrogens from clover is an important topic in respect of grazing animals, but plays a minor part for human diet, whereas exposure to phytoestrogens from soy is of higher interest, especially because the consumption of soy proteins and soy-based foods in the western population steadily increases. Vegetarians or vegans ought to ingest protein from alternative sources, e.g. in the form of soy products. Besides, a lot of formulas for

infants are soy-based [49,81], and due to the higher sensitivity of fetuses and infants, an exposure to phytoestrogens from soy is of concern.

The major isoflavone in the soy bean is genistein (Figure 7) [50]. An investigation of multiple soy-based infant formulas at the end of the 1990s showed high contents of isoflavones in these products, mostly occurring as glycosidic conjugates, when genistein was determined as major isoflavone component [49].

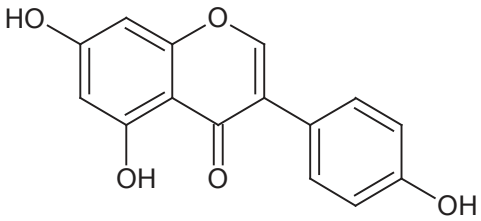

Figure 7 Chemical structure of genistein

Moreover, the concentration of the isoflavones in the plasma of four month old infants fed with soy-based infant formulas was determined, showing higher concentrations than in infants fed with cow-milk formulas or human breast milk [49]. The daily plasma concentrations were comparable to those found in adults ingesting a moderate amount of soy-containing food [49]. Such high plasma concentrations in infants allow the assumption of a high absorption and thus a high bioavailability of the isoflavones [81]. Experiments for the binding of soy isoflavones by estrogen receptors resulted in the confirmation that genistein is bound by the ER [50]. Furthermore, genistein was proven to be estrogen active *in vivo*, and to be an estrogenic development toxicant and an estrogenic carcinogen in rodents [50]. In another study, neonatal female mice were exposed to genistein on their first five days of life, resulting in an increased uterine weight after 5 days and also in abnormalities (uterine adenocarcinoma) in 18 month old mice [51]. These studies led to the assumption that genistein is also relevant in humans [50], especially when exposure to genistein occurs *in utero* or during early development. Particularly, a prenatal exposure may be of higher concern since other EAC such as DES showed effects on the offspring after *in utero* exposure (see chapter 2.2.2), why a soy-rich diet of the mother might cause problems for the offspring [81].

2.2.4 Xenoestrogens

Xenoestrogens are estrogen active natural or synthetic chemical substances that are used in products or occur in our environment. The estrogenic activity of xenoestrogens, however, cannot be predicted by means of the chemical structures of these substances, since the structures are dissimilar from the natural steroidal estrogens and also differ strongly from each other, as become clear below.

Natural xenoestrogens are – as separate group differentiated from phytoestrogens – substances from natural sources, showing estrogenic activity and occurring in the environment such as mycotoxins. The growth of mycotoxin producing fungi on cereal grains can cause problems for animal and human health, for instance resulting in reproductive and feeding disturbances in livestock cultivation [82]. The *Fusarium* toxin zearalenone (Figure 8) and some of its derivatives were proven to be mycoestrogens showing uterotrophic activity in mice [53].

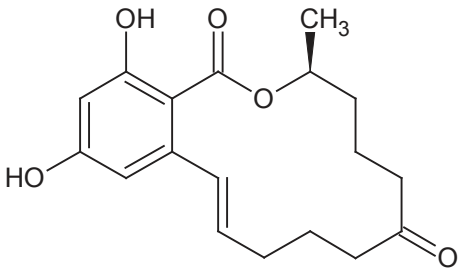

Figure 8 Chemical structure of zearalenone

Zearalenone, thus, might have an impact on humans and livestock nutrition since the mycotoxin producing fungi distribute on plants (crops) that are used for food and feed [83]. Caldwell *et al.* investigated the zearalenone production of different *Fusarium* species that were grown on autoclaved corn, and subsequently the respective ethanol extracts of the corn were tested for estrogenicity [83]. The experiments showed estrogenic activity of ethanol extracts of corn infected with *Fusarium graminearum* and of isolates of *F. tricinctum*, verified by uterine weight in a mouse bioassay [83]. Different effects to human and livestock health and various diseases due to mycotoxins and mycotoxin contaminated foods, respectively, are reported [84]. It was observed that zearalenone, which can be detected in cereals and products of cereals, was responsible for hyperestrogenism in pig that were fed with moldy grain, but it is also blamed for causing premature puberty in girls and for being responsible for cervical cancer [84]. Some studies supposed zearalenone-contaminated food as a minimal risk for human health [84], however, zearalenone might not be the only xenoestrogen exposure, and an exposure to mixtures of xenoestrogens might show

estrogenic effects even when the concentrations of individual EAC do not cause any effects [58]. Moreover, researchers in Hungary investigated patients with early breast development (telarche) and their diet, showing the presence of zearalenone both in the food and in serum samples of 14% of the patients, why a connection between contaminated food and early telarche in sensitive individuals due to chronic exposure was concluded [85].

Alongside natural xenoestrogens, wildlife and humans also can be exposed to synthetic xenoestrogens from a number of sources. At this, synthetic xenoestrogens comprise a wide range of structurally heterogeneous substances and are used, for example, in consumer products, industry or agriculture. The substances were not designed or are not used, respectively, with the aim of being estrogen active and thus to affect ecosystems or the health of humans. Examples for synthetic xenoestrogens are the plastic additive bisphenol A (BPA), pesticides such as dichlorodiphenyltrichloroethane (DDT), alkylphenolic compounds, UV filters and others. Environmental aspects, the occurrence and the effects of some synthetic xenoestrogens will be addressed below.

The pollution of waters with EAC over the whole world is reported and is steadily influenced, since EAC find their way to waters *via* landfills or waste water treatment plants but also due to environmental pollution. Thus, many studies are concerned with the impact of effluents from sewage treatment plants on aquatic organisms as described for EE2 earlier in this work (chapter 2.2.2). Studies in England and Wales, for instance, showed an increased vitellogenesis in female fish but especially high concentrations of vitellogenin in male fish exposed to the effluent of a waste water treatment plant and also in fish placed at sites of a river near the inflow of the effluent, demonstrating estrogenic contamination of sewage and water bodies [59,62].

Environmental pollution, however, not only affects aquatic systems and organisms but also other wildlife species. The impact of the insecticide DDT (Figure 9) on gull embryos, for example, was investigated by treatment of uncontaminated eggs with DDT and metabolites of DDT in levels measured in contaminated eggs in the 1970s [54]. It was observed that the treatment led to a feminization of male gulls and also to changes in the development of female embryos [54].

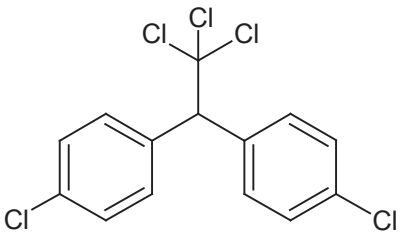

Figure 9 Chemical structure of dichlorodiphenyltrichloroethane (DDT)

In another study, DDT was injected in the yolk of eggs of domestic hens in amounts reported for eggs of wild birds, and after hatching, the hens were raised to adult age [86]. The experiments showed that the eggshells of the eggs of the hens exposed during embryonic development were significantly thinner than the eggshells of eggs of control hens, concluding that DDT exposure causes eggshell thinning [86]. Additionally, Holm *et al.* pointed out that eggs of wild birds also are subjected to further estrogenic exposure by various EAC occurring in the environment, possibly resulting in additional effects and thus in further alterations caused by endocrine disruption [86]. Moreover, a connection between the development of a declined population of brown pelicans and the DDT pollution in California around the year 1970 was presumed [87]. With a decreasing environmental concentration of DDT and its metabolites, the amount of DDT and its metabolites in anchovies – the main food of brown pelicans – decreased also, and as a consequence a recovery of the population of brown pelicans was observable [87].

The usage of DDT was limited or restricted in the 1970s in the United States of America (USA) and many other countries due to various concerns for the environment, wildlife and humans [88]. In 2001, the use of DDT was regulated with the Stockholm Convention on Persistent Organic Pollutants, which is a global treaty that came into effect in 2004 and was adopted to the European Union (EU) with the European Regulation No 850/2004/EC [89]. Thus, the use of DDT is constrained on malaria control [89]. However, since DDT and its metabolites are very persistent and accumulate in the environment and organisms, DDT is still of interest and importance today [88].

An exposure with xenoestrogens can take place through various routes, for example *via* EAC-containing products and materials or *via* the formation of EAC as breakdown products from non-estrogenic predecessors. Thus, xenoestrogens does not only concern the aquatic environment or wildlife species. An example to be mentioned is the uptake of estrogen active UV filter substances by the skin [90] or the food chain (accumulation in fish [91], accumulation in human breast milk [92]). Moreover, several studies revealed the leaching of

EAC from plastics or the accumulation of estrogen active degradation products from chemicals and the presence of these substances in food [93–95].

BPA (Figure 10), for example, is an EAC that is used as monomer for the production of polycarbonate and that is also present in epoxy resins used as coating in food cans [96]. Alongside the usage of polycarbonate for various plastic items, it is *inter alia* used for food contact materials (drinking bottles, cookware) [96], wherefore a possible leaching of BPA into food is a relevant topic. Thus, employing calculated exposure rates, food was revealed as the major source of BPA exposure [96].

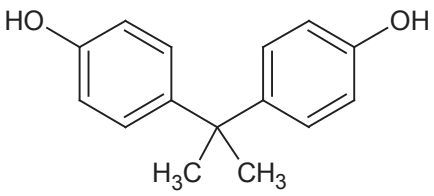

Figure 10 Chemical structure of bisphenol A (BPA)

Moreover, plastics most likely end up on landfills where critical substances can find their way into the environment, for example into leachates. Studies in Japan detected estrogenic activity of leachates and groundwater at a sea-based landfill [97]. The groundwater showed a high concentration of BPA that might have leached from plastic waste at the landfill and that was assumed to cause most of the determined estrogenicity (estimated contribution rate of 84%) [98]. Similar results have been obtained in the USA, when raw leachates of landfills were examined and showed estrogenicity, whereas the present BPA concentration was assumed to be sufficient and thus to be responsible for the observed effect [99]. Additionally, the leaching of estrogen active BPA from polycarbonate plastic tubes used in laboratory experiments by Krishnan *et al.* was detected, when the real objective actually was to determine the estrogenic activity produced by a yeast strain of *S. cerevisiae* [93].

Further xenoestrogens to be mentioned are alkylphenols. Alkylphenols (AP) are used, for example, as plastic additives [94], or as part of the production of aromatic substances (flavors, fragrances) [100]. AP, however, do more likely occur in the environment as breakdown products from alkylphenol polyethoxylates (APEO) used in many formulations such as plant protection agents (pesticides, herbicides), detergents, paints, and hair-care products [16,100]. APEO are used, for example, as nonionic surfactants in cleaning products and often end up in the aquatic environment [16]. Their degradation products are the

corresponding AP, mainly 4-nonylphenol (NP, Figure 11) and 4-octylphenol (OP, Figure 11), which particularly accumulate in sludge due to their hydrophobic properties [16]. Thus, NP is a degradation product from nonylphenol ethoxylates (NPE) used in disinfectants and in pesticides as emulsifiers [94,95].

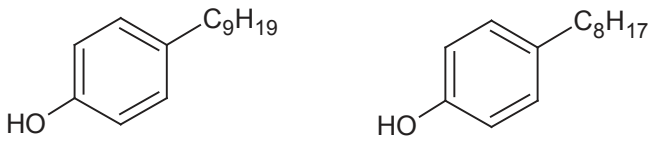

Figure 11 Chemical structures of 4-nonylphenol (NP) and 4-octylphenol (OP)

It is proven that AP are bound by the ER, and that AP can displace bound estradiol or prevent the binding of estradiol by the ER [101]. White *et al.* investigated the estrogenicity of environmentally relevant AP, while both NP and OP showed estrogenic activity in different assays, *inter alia* in studies with human breast cancer cells (MCF-7) and in an estimation of the vitellogenin production in hepatocytes from male rainbow trout using a radioimmunoassay [16]. Moreover, it has been confirmed that the activity is based on the binding by the ER, and in experiments with OP it has been shown that AP are active as estradiol mimics [16]. The estrogenic potency of surfactants and their breakdown products was also investigated by Routledge and Sumpter using a yeast estrogen screen (YES) [17] (see chapter 2.1.2). In these studies, none of the tested surfactants showed estrogenic activity, but some of the possible alkylphenolic degradation products were detected to be weak EAC [17]. In further studies employing the YES assay, Routledge and Sumpter figured out the dependence of the estrogenic activity of AP on the degree of branching in the alkyl chain, the alkyl chain length and the position of the alkyl chain [100].

As mentioned above, APEO and AP are applied in different areas, not only affecting products that might end up in the aquatic environment, but also in products with possibly direct contact to humans what might result in adverse effects on human health. NP, for example, was identified as estrogenic substance leaching from centrifuge tubes made of modified polystyrene by Soto *et al.* [94]. Additionally, NP also was reported to be ubiquitous in food, while contamination *via* different pathways is possible and varying concentrations were determined in different foodstuffs, independent of the fat content and the packaging of the food [95].

The manufacture and placing on the market of NP and NPE are regulated in the EU by Regulation No 1907/2006/EC [102]. This so-called REACH Regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) restricts the usage and placing to the market of NP and NPE as single substances or in mixtures to an amount of at most 0.1% by

weight, valid for specific products and purposes given in Annex XVII of this regulation, including cosmetics, personal care products and different kinds of cleaning processes [102]. Despite this regulation, NP is still found in the environment and therefore needs further attention.

Other critical substances with estrogenic potential are organic filter substances used in UV screens and other cosmetics or technical products. These chemicals are allowed to be added to cosmetic products [103], on the one hand to sun screens for skin protection (main usage), on the other hand in order to protect further ingredients of the cosmetic products against UV radiation. Since cosmetics usually have direct contact to the human skin and UV filter substances could accumulate in human and wildlife due their relatively high lipophilicity, the estrogenic activity of these substances is of concern.

The organic UV filters benzophenone-3 (Figure 12) and octyl methoxycinnamate (Figure 12), for example, were detected in human breast milk from 5 of 6 tested mothers in Germany in the 1990s, indicating accumulation of these chemicals in the adipose tissue [92].

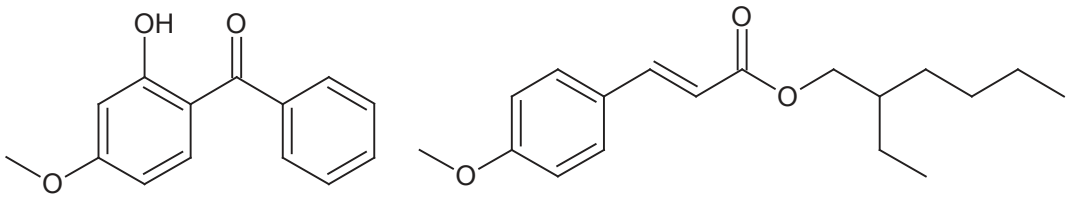

Figure 12 Chemical structures of benzophenone-3 and octyl methoxycinnamate

Moreover, in the years of 1991 and 1993, up to six different UV filter substances were detected in fish (European perch and roach) of the Meerfelder Maar (maar in the Eifel in the West of Germany), including benzophenone-3, octyl methoxycinnamate and 4-methylbenzylidene camphor (Figure 13) [91]. The water of this maar was also investigated, however, only in one year a single substance was detectable, why a high bioaccumulation of these substances in fish was concluded [91]. Since the maar is commonly used as lake for bathing, the bathers were the most likely reason for the contamination and the subsequent accumulation of the UV filters in fish [91].

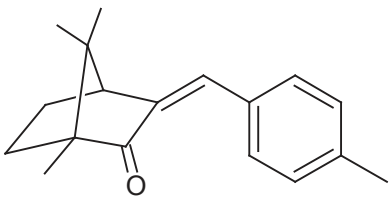

Figure 13 Chemical structure of 4-methylbenzylidene camphor

Studies on the estrogenicity of 6 UV filter substances were executed in an *in vitro* assay (E-Screen with MCF-7 breast cancer cells) and *in vivo* with an uterotrophic assay [18]. The *in vitro* test showed estrogenic activity of 5 of 6 compounds tested, including benzophenone-3, octyl methoxycinnamate and 4-methylbenzylidene camphor [18], which are two of the UV filters detected in human breast milk [92] and three of the UV filters detected in fish [91]. These three UV filters also showed an increased uterine weight in the uterotrophic assay in the immature rat after oral administration, indicating *in vivo* estrogenicity [18]. However, it was pointed out that it is uncertain whether the compounds themselves or possible metabolites caused the effects observed [18]. Despite their *in vivo* and *in vitro* estrogenicity, the mentioned UV filters are allowed for usage in cosmetic products, regulated by the EU in Regulation No 1223/2009/EC [103]. In the ready to use preparation, benzophenone-3 and octyl methoxycinnamate are allowed up to a maximum concentration of 10%, and 4-methylbenzylidene camphor up to 4% [103]. Experiments by Janjua *et al.* demonstrated, moreover, the dermal adsorption of the UV filters benzophenone-3, octyl methoxycinnamate and 4-methylbenzylidene camphor, which were all found in plasma and urine samples, when postmenstrual women and young men were exposed with lotion (topical administration) containing all three of these substances [90]. Additionally, reproductive hormone levels were investigated but no effects were observed [90]. Despite these observations, it has to be in mind that the same exposure might affect the endocrine system of children, since children have lower levels of endogenous hormones and also the elimination way for drugs is not as developed as in adults, why their endocrine system can be disrupted easier [90].

Last but not least, parabens (Figure 14), *i.e.*, parahydroxybenzoates or esters of parahydroxybenzoic acid, shall be mentioned as example for widely used substances showing estrogenic activity.

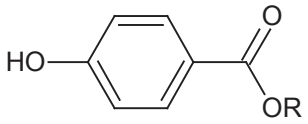

Figure 14 Chemical structure of parabens (R = alkyl group)

Parabens show alkyl chain length dependent antimicrobial activity [104], and are used as preservatives in cosmetics and food, and also in medication, thus resulting in direct exposure to humans. Maximum concentrations for the use in cosmetic products are given in Regulation No 1223/2009/EC of the European Parliament and the Council [103]. In the ready to use preparations, 4-hydroxybenzoic acid and its salts and esters (methyl, ethyl, propyl, butyl) are allowed with a maximum concentration of 0.4% (as acid) for single esters and

0.8% (as acid) for mixtures of esters, respectively [103]. Ethyl and methyl derivatives of parahydroxybenzoic acid are, moreover, allowed as food additives with varying maximum levels according to respectively specified food categories, regulated by Regulation No 1333/2008/EC on food additives [105]. In a work by Routledge *et al.* [104], different parabens were tested for estrogenicity using a YES assay according to [17]. Methyl, ethyl, propyl and butyl derivatives all showed estrogenic activity in the YES assay, whereas parahydroxybenzoic acid, which is the parent substance of the mentioned derivatives and their main metabolite, was inactive [104]. The estrogenicity increased with the alkyl chain length, and the methyl and butyl esters were additionally tested in two different *in vivo* tests (uterotrophic assays in rats) [104]. After subcutaneous injection, methyl paraben did not increase the uterus weight, whereas butyl paraben led to an uterotrophic response [104].

2.3 Brief summary on EAC

The preceded sections gave an overview of the broad spectrum of substances showing estrogenic activity, the possible ways for an exposure to EAC, and their effects. It becomes apparent that estrogenicity is not limited to natural hormones or substances with structural similarities to these, but that EAC are structurally very heterogeneous and an exposure through many different routes is possible.

Therefore, an environmental monitoring in terms of EAC, especially a monitoring of the aquatic system, is meaningful in order to estimate current estrogenic load or, for example, to verify the efficiency of waste water treatment. Apart from environmental aspects, nowadays, there is also an increasing interest in products that not contain consciously added substances identified to be estrogenic or endocrine active such as parabens in cosmetics, what frequently is also highlighted in product advertisements.

For a monitoring, different tests are available, including various *in vivo* and *in vitro* tests indicating estrogenic activity. Standard chromatographic methods, moreover, can be used for the determination of specific substances with known endocrine disrupting properties. The planar yeast estrogen screen provides, however, the separation, detection and determination of individual components, including an evidence of estrogenic activity, in a single test. And therefore, the pYES is a very suitable tool for environmental samples to monitor whether the samples contain estrogenic compounds, and additionally to identify and quantify these substances.

3 Legislative aspects in respect of waters (European Union)

Considering the chemical load of the aquatic environment due to emission of hazardous substances, and thus health and population risks especially for aquatic organisms, and in respect of the reduction of the pollution, the European Commission worked out different legislations. The EU Water Framework Directive (Directive 2000/60/EC), which is the main water policy instrument for setting antipollution strategies, amended by the decision and directives given in [106–108], orders the member states to conduct necessary steps to progressively reduce the pollution by priority substances and to (stepwise) terminate emissions, discharge and loss of these substances [109]. In this regard, priority substances are substances that cause a high risk for or *via* the aquatic environment. Thus, Directive 2008/105/EC (amended by Directive 2013/39/EU [108]) lists environmental quality standards (EQS) for pollutants classified as priority substances according to the Water Framework Directive [109], primarily for inland and other surface waters, but due to the potential of an accumulation also sediments and biota ought to be under investigation [107]. The list of priority substances in the field of water policy is given in Annex X of Directive 2000/60/EC, and indicates *inter alia* nonyl- and octylphenols, while nonylphenols are stated as identified priority hazardous substances [107–109]. Annex I of Directive 2008/105/EC lists the EQS of the priority substances and certain other pollutants with maximum allowable concentrations in inland and other surface waters, but also in biota, where biota means fish if not otherwise indicated [107,108]. Amongst 45 substances or substance groups, this list includes DDT, NP and OP (4-*tert*-OP) [108]. Article 8b of Directive 2008/105/EC implies to the Commission to create a watch list of substances that are likely to be a considerable risk to or *via* the aquatic environment and that should be monitored due to the lack of monitoring data in order to support future processes of prioritization [107]. The list comprises 10 substances or groups of substances, the respective matrices and also analytical methods for the determination of the substances or groups of substances with respective maximum acceptable method detection limits, implemented by the Commission Implementing Decision 2015/495/EU [110]. The watch list *inter alia* includes the natural estrogens E2 and E1, the synthetic estrogen EE2 and the estrogen active UV filter 2-ethylhexyl-4-methoxycinnamate (octyl methoxycinnamate) [110]. Maximum acceptable detection limits for the natural and synthetic estrogens are 0.4 ng/L for both E2 and E1 and 0.035 ng/L for EE2, while the analytical methods indicated are large-volume solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS-MS) for EE2 and SPE–LC-MS-MS for both E2 and E1 [110]. The method indicated for octyl methoxycinnamate is SPE–LC-MS-MS or gas chromatography MS with a maximum acceptable detection limit of 6000 ng/L [110].

4 Scope and aims of this work

Regarding the ubiquitous presence of EAC in our environment and especially the risks for wildlife and humans, and with respect to the watch list of the Implementing Decision of the European Commission, it is of great importance to monitor EAC in environmental samples.

The aim of his work was to develop a planar yeast estrogen screen (pYES, or high-performance thin-layer chromatography-planar yeast estrogen screen, HPTLC-pYES) as a screening for EAC in water and sewage samples. The pursued objective was the development of a method that allow a bioautography for EAC after prior chromatography directly on the HPTLC plate employing the recombinant yeast strain generated by McDonnell *et al.* [34,35]. The yeasts contain the human estrogen receptor and the *LacZ* reporter gene encoding for the enzyme β-galactosidase [34,35]. Since many environmental samples contain components showing native fluorescences (blue, red), the detection of blue fluorescing methylumbelliferone, which is enzymatically released from the generally used substrate 4-methylumbelliferyl-β-D-galactopyranoside, can be interfered. It was purposed to introduce a substrate, which allows the differentiation of native fluorescences and positive pYES-signals caused by estrogenic activity. Additionally, it had to be verified, whether HPTLC simultaneously represents a planar clean-up since environmental samples are commonly highly polluted, thus often require complex extraction and purification steps such as a solid phase extraction (SPE).

Furthermore, the pYES to be developed should enable the quantitation of EAC and the identification of unknown EAC by means of coupling HPTLC to mass spectrometry. In addition, the application of pYES on further samples should be tested, e.g. on food samples.

Since receptor assays such as the pYES usually show a saturation behavior (exemplarily shown for the YES assay in [17,111]), *i.e.*, provide sigmoidal dose-response curves on a logarithmical scale, a logit-log evaluation of pYES ought to be possible. Therefore, another objective was to proof this assumption and to transfer the logit-log method as evaluation tool to pYES (normally used in microtiter plate assays such as radioimmunoassays and enzyme-linked immunosorbent assays [112–115]). Dose-response curves additionally will allow the determination of half maximal effect doses (ED_{50}) [33] and therefore the determination of estradiol equivalent factors (EEF) of known EAC, and the estradiol equivalent concentration (EEQ) or estradiol equivalent amount (EEA) of known and unknown EAC in liquid or solid samples.

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II Publications

Publication 1

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Planar yeast estrogen screen with resorufin-β-D-galactopyranoside as substrate

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ABSTRACT

For the planar yeast estrogen screen (pYES), 4-methylumbelliferyl-β-D-galactopyranoside was generally employed as substrate, delivering blue fluorescing 4-methylumbelliferone after enzymatic cleavage by the YES reporter β-D-galactosidase as the positive signal for the presence of estrogen active compounds (EAC). As environmental samples like waste water also contain blue fluorescent components, it is difficult to differentiate them from pYES signals. Therefore, resorufin-β-D-galactopyranoside (RGP), providing the orange fluorescing resorufin after enzymatic cleavage, was introduced as pYES substrate to determine EAC. With 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), mean limits of detection and quantitation of 3.5 and 6.5 pg/zone, respectively, were determined. Obtained recoveries for both E2 and EE2 from spiked water samples in a concentration range of 2–20 ng/L were close to 100%. The application of the RGP-pYES on waste water influent and effluent samples showed the clear detection of EAC without interferences. Estrone (E1), Estriol, E2, and an unknown EAC were found in the influent sample (E2 with a mean of 16.9 ng/L and a precision of 11% RSD; n = 4), while another unknown EAC was observed in the effluent sample. In addition, the presence of conjugated EAC in the influent was demonstrated by hydrolysis with β-glucuronidase, when the signals of E1 and the unknown increased by about 25% and 100%, respectively.

1. Introduction

The European Commission published an implementing decision [1] to establish a watch list of substances for Union-wide monitoring in the field of water policy according to a preceding directive [2,3]. The monitoring shall help to identify the actual risk posed by the listed substances. This watch list includes the estrogen active compounds (EAC) 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1). In addition, possible suitable methods of analysis including maximum acceptable detection limits are listed. Method detection limits should be at least as low as the presumable no-effect concentration of each substance and should be lowered when new information shows a decrease of the no-effect concentration. According to the implementing decision of March 2015 [1], the maximum acceptable method detection limits are listed as follows: 0.4 ng/L for E2 and E1, and 0.035 ng/L for EE2. The indicated methods include a solid-phase extraction and a large volume solid-phase extraction for E2/E1 and EE2, respectively, followed by liquid chromatography-tandem mass spectrometry.

In the field of trace analysis of EAC, different *in vitro* methods has gained great importance, like the yeast estrogen screen (YES) [4], the estrogen responsive chemical activated luciferase gene expression assay (ER-CALUX) [5], or the estrogen screen (E-screen) [6], which are performed in microtiter plates. The YES assay was also successfully combined with planar chromatography (planar YES, pYES) providing a chromatographic separation of samples by high-performance thin-layer chromatography (HPTLC) prior to bio-detection by the yeast cells [7–12]. Initially, pYES was performed on normal phase silica HPTLC plates resulting in broadened zones after humid incubations, which were difficult or impossible to quantify [7–9]. This problem was solved by application of water-wettable reversed phase plates (RP–18 W) providing sharp-bounded zones, even after prolonged incubation in an humid atmosphere [10]. Different applications of the method proved the progress [11,12]. The generally used substrate for pYES was 4-methylumbelliferyl-β-D-galactopyranoside (MUG) that delivers blue fluorescing 4-methylumbelliferone (MU) after enzymatic cleavage by the YES reporter β-D-galactosidase as the positive signal for the presence of an EAC in an HPTLC zone. However, many environmental samples, like influents or effluents of waste water treatment plants, pore water of sediments, and even surface waters, or herbal extracts, originally contain blue fluorescent compounds that can interfere the detection of MU [11,12]. To differentiate

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native fluorescences from the fluorescence arising from estrogenic activity by pYES, the application of another substrate than MUG is greatly advantageous. Therefore, resorufin-β-D-galactopyranoside (RGP) was introduced as substrate, delivering orange fluorescing resorufin and allowing the detection of EAC without interfering signals due to blue or red fluorescing compounds in a sample. To receive optimal conditions for the pYES test an adjustment of the pH of the HPTLC plate was required. After the development of the modified pYES, the system was tested by determining E2 and EE2 in spiked water samples. The applicability and precision of the method was additionally demonstrated by studying waste water samples. Since hormones are conjugated in the human body for excretion [13], the waste water samples were also incubated with β-glucuronidase and compared with untreated samples.

2. Materials and methods

2.1. Chemicals and materials

Yeast nitrogen base without amino acids (YNB) for culture media was purchased from Sigma-Aldrich (Steinheim, Germany), YNB for agar plates, agar, adenine, and casamino acids [14] were from Becton Dickinson (Heidelberg, Germany). L-lysine (≥98%), magnesium sulfate heptahydrate (puriss. p.a.), sodium sulfate (anhydrous, >99%), *n*-hexane (for pesticide residue analysis) and ethyl acetate (for pesticide residue analysis) were from Fluka-Sigma-Aldrich (Steinheim, Germany). Ethanol (≥99.8%), methanol (≥99.9%), *t*-butyl methyl ether (TBME, ≥99.8%), β-galactosidase (from *Aspergillus oryzae*), β-glucuronidase (type H-2 from *Helix pomatia*), 17β-estradiol (E2, ≥98%), 17α-ethinylestradiol (EE2, ≥98%), estrone (E1, ≥99%), and estriol (E3, ≥97%) were purchased from Sigma-Aldrich. The following substances and materials were from Merck (Darmstadt, Germany): L-histidine (≥99%), copper(II) sulfate pentahydrate (p.a.), potassium chloride (>99%), disodium hydrogen phosphate (p.a.), sodium acetate (anhydrous for analysis), potassium dihydrogen phosphate (p.a.), hydrochloric acid (37%), sodium hydroxide solution (20%) and HPTLC glass plates silica gel 60 RP–18 W (200 mm × 100 mm, layer thickness 200 μm). D-Glucose (anhydrous) and sodium hydrogen carbonate were from BDH Prolabo Chemicals (VWR, Bruchsal, Germany). Resorufin-β-D-galactopyranoside (RGP) and β-estradiol-17-(β-D-glucuronide) sodium salt were obtained from Santa Cruz biotechnology (Dallas, USA). Dimethyl sulfoxide (DMSO, 99.98%) was from Fisher Scientific (Schwerte, Germany), and sodium chloride (≥99%), acetone (≥99.8%) and acetic acid (100%) were from Carl Roth (Karlsruhe, Germany). Ultrapure water was produced by a Synergy System (Millipore, Schwalbach, Germany). The used recombinant yeast strain was *Saccharomyces cerevisiae* BJ3505 (protease deficient, MATα, PEP4:HIS3, prb1-Δ1.6R, HIS3-Δ200, lys2-801, trp1-Δ101, ura3-52gal2can1) and was generated by McDonnell et al. [15,16]. The yeasts were stored in a cryo-vial at −70 °C. Orange filter glass O 580 was from HEBO (Aalen, Germany) and was cut to shape as optical filter for the TLC Scanner 4 (CAMAG, Muttenz, Switzerland). The pH values of the HPTLC plates were measured by a pH meter EL20/EL2 from Mettler Toledo (Columbus, USA) with a surface electrode SenTix Sur from WTW (Weilheim, Germany) using automatic endpoint format. HPTLC plate incubations were performed in a box with approximately 100% relative humidity.

2.2. Stock and standard solutions

Standard stock solutions of E2 and EE2 were prepared in ethanol (1 mg/L). Standard solutions for calibration were obtained by diluting the stock solutions with ethanol to concentrations of 1, 10 and 100 μg/L. Solutions of E1 (200 μg/L) and E3 (500 μg/L) were prepared in ethanol. A stock solution of β-estradiol-17-(β-D-glucuronide) was prepared in DMSO (60 mg/L) and was diluted with ultrapure water to a concentration of 20 ng/L. Stock solutions were stored at −20 °C.

2.3. Media and agars

Growth medium for overnight cultures was composed of 6.8 g/L YNB, 1 g/L glucose, 170 mg/L L-lysine and 100 mg/L L-histidine. The solution of YNB plus amino acids and the solution of glucose were sterilized separately by steam (120 °C, 20 min) and were mixed after autoclaving. Test medium for yeast incubations moreover contained 112 μM $CuSO_4$. Plate agar consisted of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L) and agar (20 g/L).

2.4. Strain maintenance and cultivation of yeast cells for the pYES (test culture)

To obtain isolated colonies of yeast cells, the cell mass of a cryo-vial (stored at −70 °C) was streaked in a three-phase streaking pattern on an agar plate that was incubated at 30 °C for at least 48 h followed by storage at 2–8 °C for a maximum of 2 months. About 50 mL of growth medium were placed into a 250-mL shaking flask, inoculated with a single colony from the agar plate and incubated at 30 °C overnight with constant shaking at 150 rpm (incubating mini shaker, orbit 3 mm, VWR, Darmstadt, Germany). An aliquot (2 mL) of an overnight culture was transferred into 50 mL of fresh growth medium and cultured overnight (30 °C, 150 rpm) to get a fresh culture for the next day (repeatable for five days). The number of cells was measured with a TC20 automated cell counter (Bio-Rad Laboratories, Munich, Germany). For pYES, a cell number of 6–8 × 10^7 cells/mL was needed. For this purpose, the required volume of overnight cultures was centrifuged (2576*g*, Biofuge primo R, Heraeus, Hanau, Germany) for 5 min at room temperature, the supernatant was discarded and the remaining cell pellets were re-suspended in 40 mL of test medium (for glass dipping chamber for 10 cm × 10 cm plates, biostep, Burckardtsdorf, Germany). Flasks for cultivation were without baffles and were sterilized by steam at 120 °C for 20 min before use.

2.5. Substrate solution

A stock solution of resorufin-β-D-galactopyranoside (RGP) with a concentration of 20 mg/mL in DMSO was prepared and stored at −20 °C. The substrate dipping solution (0.1 mg/mL) was obtained by diluting the stock solution (200 μL) with phosphate buffer (40 mL) consisting of KH_2PO_4 (40.8 g/L), Na_2HPO_4 (42.6 g/L), $MgSO_4 \cdot 7H_2O$ (1.2 g/L) and KCl (3.7 g/L), adjusted to pH 7 with sodium hydroxide solution (20%).

2.6. Planar yeast estrogen screen (pYES)—general procedure

HPTLC glass plates silica gel 60 RP–18 W were prewashed with acetone/water (9/1, v/v) and dried for 30 min at 120 °C on the TLC Plate Heater III (CAMAG). The cooled down plates were dipped with the chromatogram immersion device (CAMAG, time 3 s, speed 3) into a solution of sodium hydrogen carbonate (25 g/L) adjusted to a pH of 6.4 with hydrochloric acid (37%) and dried in a cold airstream for 5 min. Sample applications were performed with an Automatic TLC Sampler 4 (ATS 4, CAMAG) at 10 mm from both the lower edge and the left side. The rinsing solvent was ethanol. Extracts of water samples (50 μL) were applied onto 10 cm × 10 cm HPTLC plates as 5 mm × 10 mm areas, and different volumes of the standard solutions as 5 mm bands, resulting in 3, 5, 10, 50, 100, and 200 pg E2 and EE2 per band (track distance set to automatic). The plate was

first developed with methanol up to 15 mm to focus the application areas and dried for 2 min in a cold airstream. The second plate development was performed with *n*-hexane/toluene/ethyl acetate (4/1.5/2, v/v/v) [12] up to a migration distance of 70 mm. The developments were carried out in a twin-trough chamber (10 cm x 10 cm, CAMAG). Subsequently, the plate was dried for two minutes in a cold airstream, immersed with the chromatogram immersion device (time 3 s, speed 3) into a test culture adjusted to 6-8 $\times$ 10^7 cells/mL and left in the vertical position for 30–60 s for uniform distribution of yeast cells. The plate was incubated in a box (relative humidity, RH ~ 100%, 30 °C) for 4 h. After drying in a cold airstream for five minutes, the plate was dipped into RGP substrate solution (0.1 mg/mL, time 3 s, speed 3) and incubated at 37 °C in a box with approximately 100% RH for 30 min. Drying, dipping and incubation was repeated two times. Plate images were captured under UV 254 nm and UV 366 nm (TLC Visualizer, CAMAG), and the plate was scanned in fluorescence mode at 550/ > 580 nm using the tungsten lamp (TLC Scanner 4, CAMAG). HPTLC instruments were controlled by the software winCATS, version 1.4.6 (CAMAG).

2.7. Optimization of substrate incubation

An aqueous solution of β-galactosidase (1 mg/mL) was applied (1 μL, 5 mm bands) onto 10 tracks (distance 20 mm) of six HPTLC plates (20 cm × 10 cm). The plates were cut into 2 cm × 10 cm sections (smartcut plate cutter, CAMAG). Half of the plate stripes were immersed into a substrate solution of RGP (0.1 mg/mL, time 3 s, speed 3) and incubated at 37 °C (without box) for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min (n = 3). The other plate stripes were dipped in substrate solutions of various concentrations of RGP (0.01, 0.02, 0.04, 0.06, 0.1, 0.15. 0.2, 0.25, 0.35 and 0.5 mg/mL) and incubated for 50 min at 37 °C (without box; n = 3).

2.8. Optimization of yeast incubation

A standard solution of E2 (1 μg/L) was applied (10 μL) onto 10 tracks (5 mm bands, distance 20 mm) of eight HPTLC plates (20 cm × 10 cm). The plates were cut into 2 cm × 10 cm stripes, which were dipped into yeast cell suspensions of different cell density (time 3 s, speed 3). After incubation at 30 °C in a box (RH ~ 100%) for 3 h, the stripes were dried in a cold airstream for five minutes. The plate stripes were dipped into a RGP substrate solution (0.1 mg/mL, time 3 s, speed 3) and incubated (30 min, 37 °C, without box). Drying, dipping and incubation were repeated two times. In addition, 10 pg of E2 (10 μL of a 1 μg/L solution, 5 mm bands, distance 20 mm) were applied onto 10 tracks of two HPTLC plates (20 cm × 10 cm). After cutting the plates into 2 cm × 10 cm sections, the stripes were dipped in a test culture with a cell density of 6–8 × 10^7 cells/mL and incubated at 30 °C in a box (RH ~ 100%) for 2, 3, 4, 5, and 6 h (n = 4). Thereafter, the plate stripes were dried for 5 min in a cold airstream, and substrate incubation was performed as described above.

2.9. Limits of detection and quantitation (LOD/LOQ)

For the determination of LOD and LOQ, 1–10 μL of a 1 μg/L standard solution of E2 and EE2 were applied onto 10 tracks of 10 cm × 10 cm HPTLC plates as 5 mm bands. The plates were developed in a twin-trough chamber with *n*-hexane/toluene/ethyl acetate (4/1.5/2, v/v/v) [12] up to a migration distance of 70 mm. Yeast incubation, substrate incubation, and plate evaluation were performed as described above (2.6 pYES general procedure).

2.10. Sample extraction and deconjugation of EAC in waste water

The extraction and the following pYES were tested with aqueous solutions of E2 and EE2 at four different levels of 2, 6, 14, and 20 ng/L. Aliquots of the spiked samples (30 mL) were transferred to 40-mL centrifuge tubes containing 6 g of sodium chloride. TBME (5 mL) was added, and the tubes were closed and placed on an orbital shaker (KS125 basic from IKA, Staufen, Germany; orbit 4 mm) for 30 min at 800 rpm. The organic phase was separated in a MIXXOR liquid–liquid extraction system (Lidex Technologies, Bedford, USA), evaporated by a stream of nitrogen, and the residue was dissolved in 200 μL of ethanol. In addition, ultrapure water was extracted in the same way to obtain a blank extract. The extracts were stored at −20 °C until analysis.

Waste water samples were taken from a local waste water treatment plant (Treatment Plant for Education and Research, in the west of the city Stuttgart, Germany). The plant treats the daily waste water of about 10,000 inhabitants corresponding to about 2000 m^3 of sewage daily. Influent and effluent samples were taken in the morning (April 2016), and were frozen until analysis to prevent oxidation of E2 [13]. After thawing, the sewage water samples were extracted in the same way as the spiked samples. However, the obtained extract (TBME) volume was determined with a measuring cylinder with graduation divisions of 0.1 mL, and the application volumes (pYES) were adapted with regard to the obtained TBME volume of a blank extract. In case of an occurring emulsion layer after shaking (observed for influent samples), the emulsion and the TBME phase were separated from the aqueous phase by removing the lower water phase with a pipette. Sodium sulfate (anhydrous) was added until the emulsion was broken, and the received TBME volume was determined. TBME was removed by a stream of nitrogen, and the residue was dissolved in 200 μL of ethanol. The extracts were stored at −20 °C until analysis. Additionally, the waste water samples were treated with a solution of β-glucuronidase (type H-2 from *Helix pomatia*, ≥85,000 units/mL, with additional sulfatase activity ≤ 7500 units/mL) to hydrolyze possibly present conjugated EAC. Therefore, 30 mL of the sewage samples were mixed with 3 mL of 1 M sodium acetate buffer (pH 5.5) and subsequently with 10 μL of the 10-fold diluted enzyme solution. Incubation was performed at 37 °C for 16 h. After cooling down, the extraction was performed as described for the untreated waste water samples. Additionally, the efficiency of the enzyme solution was tested with a water sample spiked with β-estradiol-17-(β-D-glucuronide) (20 ng/L), and 'hydrolyzed blanks' were also extracted, when the acetate buffer and the enzyme solution were added to ultrapure water, followed by incubation.

3. Results and discussion

3.1. pH adjustment of HPTLC plates

The advantageous application of HPTLC glass plates silica gel 60 RP–18 W to obtain sharp-bounded zones despite the humid conditions during the pYES assay has already been demonstrated, when it also was shown that the acidic plate material requires a pH adjustment to get a detectable signal of MU [10]. The pH adjustment was achieved with a substrate solution of MUG in citric buffer with a pH of 12 and an additional stop solution of pH 12 to terminate the substrate reaction [10]. For the planar YES in the present work, the recombinant yeast strain *Saccharomyces cerevisiae* generated by McDonnell et al. [15,16] was used. An expression vector for the human estrogen receptor (hER) was constructed and inserted in the yeast [15]. Also, a reporter plasmid was created which consists of the yeast iso1-cytochrome *c* proximal promoter elements which are fused to the structural gene for β-galactosidase from

Table 1
Mean pH values of HPTLC plates RP-18 W measured with a surface electrode (n = 6); sodium hydrogen carbonate solution: 25 g/L, pH 6.4; incubation with test culture 3 h, 30 °C, closed box, RH ~ 100%; substrate incubation: substrate RGP in phosphate buffer (0.6 M, pH 7), 37 °C, 30 min.

Treatment	mean pH value ± SD[a]
Without pretreatment	4.7 ± 0.3
After treatment with sodium hydrogen carbonate solution	6.5 ± 0.3
After incubation with test culture	5.8 ± 0.2
After first substrate incubation	6.5 ± 0.1
After second substrate incubation	6.6 ± 0.1
After third substrate incubation	6.7 ± 0.2

[a] standard deviation.

Escherichia coli (*lacZ*-CYC fusion) [15,17,18]. By binding a ligand, the transcription of the *lacZ* reporter gene is induced and thus the production of β-galactosidase [16]. With regard to the integrated reporter plasmid, the optimal pH range for the β-galactosidase had to be considered. The maximum activity of β-galactosidase from *Escherichia coli* with o-nitrophenyl-β-D-galactoside as substrate was obtained at a pH 7.2-7.3, and the enzyme was stable in a pH range of 6–8 [19]. Therefore, the pH of the HPTLC plate should be above 6 to ensure the optimal cleavage of the substrate by the enzyme. The pH span for optimal growth and metabolism of yeasts was reported between 4.5 and 6.5 [20], wherefore such a pH range should be achieved on the plate during incubation with the test culture. On average, the measured pH of the HPTLC RP–18 W plate was below 5 (Table 1). This would be a fair pH for yeast incubation, but far away from the ideal pH for β-galactosidase activity. As resorufin-β-D-galactopyranoside (RGP) is not stable at a pH of 12 (the solution showed a dark coloration within a few minutes), another way had to be found to adjust the pH of the plate layer to the requirements of both yeast cells and β-galactosidase. Thus, the pH of the prewashed plates was adjusted to >6 by dipping them into a solution of sodium hydrogen carbonate (Table 1). During yeast incubation, the pH decreased to just below 6 (Table 1). Hence it was possible to use a substrate solution buffered to pH 7, like reported in literature [7,8], to raise the pH after yeast incubation above 6, but the buffer concentration was chosen to 0.6 M to really ensure the pH increase for the enzyme reaction. Thus, HPTLC plates were dipped into substrate solution (0.1 mg/mL RGP in 0.6 M buffer) and incubated at 37 °C for 30 min. After the incubation, the plates were dried, dipped again into substrate solution and incubated again. This step was repeated a third time. After the three-fold substrate incubation, the measured pH on the plate was between 6.5 and 7 (Table 1). Triple immersion not only increased the pH value and thus the signal, but also resulted in more homogenous distribution of the substrate on the plate. Enzymatic hydrolysis of the substrate RGP releases resorufin, which is fluorescent only in its anionic form, but has a relatively low pK_a of 5.8 [21]. Consequently, 80–90% of the resorufin should be present in the deprotonated form at the plate pH determined after triple substrate incubation. Attempts have been made to raise the pH after the substrate incubation even further to obtain more molecules in the anionic form. Therefore, plates were dipped in different carbonate buffers (0.1 M, pH 10 and 11; 0.3 M, pH 10; 0.5 M, pH 9 and 10; 1 M, pH 9 and 10), which, however, did not improve signal intensities or even resulted in damage of the plate layer (blisters, flaking off).

3.2. Substrate incubation

To determine the optimal parameters for the substrate reaction, substrate solutions of varying concentrations and different periods of substrate incubation were examined, when all incubations were performed at 37 °C according to the temperature given in literature [7,8,10]. Therefore, the same amount of β-galactosidase was multiply applied on different plate stripes. For the determination of the optimal incubation time, a substrate solution of 0.1 mg/mL RGP was used based on data reported in literature (RGP used in a flow cytometric assay [22]). The maximum signal was obtained at an incubation time of 50 min (Fig. S1). However, since already 96% of the maximum signal were reached after 30 min of incubation, the incubation time for the substrate reaction was defined to 30 min. When plotting the measured signals against the associated concentrations of RGP, a curve was observed that showed a high slope at the beginning and started to flatten at concentrations above 0.06 mg/mL (Fig. S2). At 0.2 mg/mL RGP, about 90%, and at 0.1 mg/mL, about 80% of the signal of the highest examined concentration of 0.5 mg/mL were reached. However, the formation of a whitish precipitation was observed in solutions of 0.2 mg/mL RGP or higher concentrations after storage at −20 °C that did not resolubilize and that remained on the plate afterwards. Therefore, the optimal concentration of the substrate solution was defined to 0.1 mg/mL RGP.

3.3. Cell density and incubation time with yeast cells

To find out the optimal cell density for pYES, E2 (10 pg) was multiply applied on different plate stripes, which were dipped into test cultures of cell densities up to 5.5 x 10^7 cells/mL and were incubated for 3 h at 30 °C (RH ~ 100%). An incubation time of 3 h was chosen based on literature [10]. At cell densities above 1.0 × 10^7 cells/mL, at first it seemed that saturation was reached (Fig. S3). At cell densities higher than 2.8 x 10^7 cells/mL, however, a further increase of the fluorescence signal was observed. Therefore, test cultures with cell densities up to 1.5 × 10^8 cells/mL were prepared. The experiment was repeated five times on five different days with both a new test culture and HPTLC plate (each plate pretreated with a freshly prepared solution of sodium hydrogen carbonate). The resulting fluorescence signal increased with the increasing number of cells, passing a maximum between about 6 × 10^7 and 1 × 10^8 cells/mL (Fig. S4). Therefore, a span of about 6 × 10^7 to 8 × 10^7 cells/mL was chosen as optimal cell densities for further experiments.

To determine the optimal incubation time with yeasts, again 10 pg of E2 were applied on plate stripes, which were dipped into a cell suspension of 6–8 × 10^7 cells/mL and incubated for different times from 2 to 6 h (30 °C, RH ~ 100%). The fluorescence signal of resorufin increased strongly during the first 3 h until the curve flattened at an incubation time of 4 h (Fig. S5), when also the standard deviations became rather low. Thus, it was assumed that a stable expression of β-galactosidase was reached and the optimal incubation time with yeast cells was defined to 4 h.

3.4. pYES

The final conditions for the pYES, sketched in Fig. 1, were chosen based on the results of the executed experiments. The HPTLC plates were pretreated, extracts and standard solutions were applied and the analytes were separated by chromatography. Cell density for yeast incubation was adjusted to 6–8 x 10^7 cells/mL. The plates were left in a vertical position after immersion in test culture for 30–60 s to obtain a uniform distribution of yeast cells on the plate. Yeast incubation was performed in a closed box at approximately 100% RH at 30 °C for 4 h. After incubation with yeast cells, the substrate incubation was performed (0.1 mg/mL RGP in 0.6 M phosphate buffer, pH 7). For pH enhancement and thus the signal intensity, and to obtain an even background, the substrate incubation was executed in a closed box (RH ~ 100%) three times at 37 °C for 30 min. Orange fluorescence of the released resorufin was documented under UV 254 nm and 366 nm and the plates were scanned at 550 nm with an optical filter (>580 nm) in fluorescence mode.

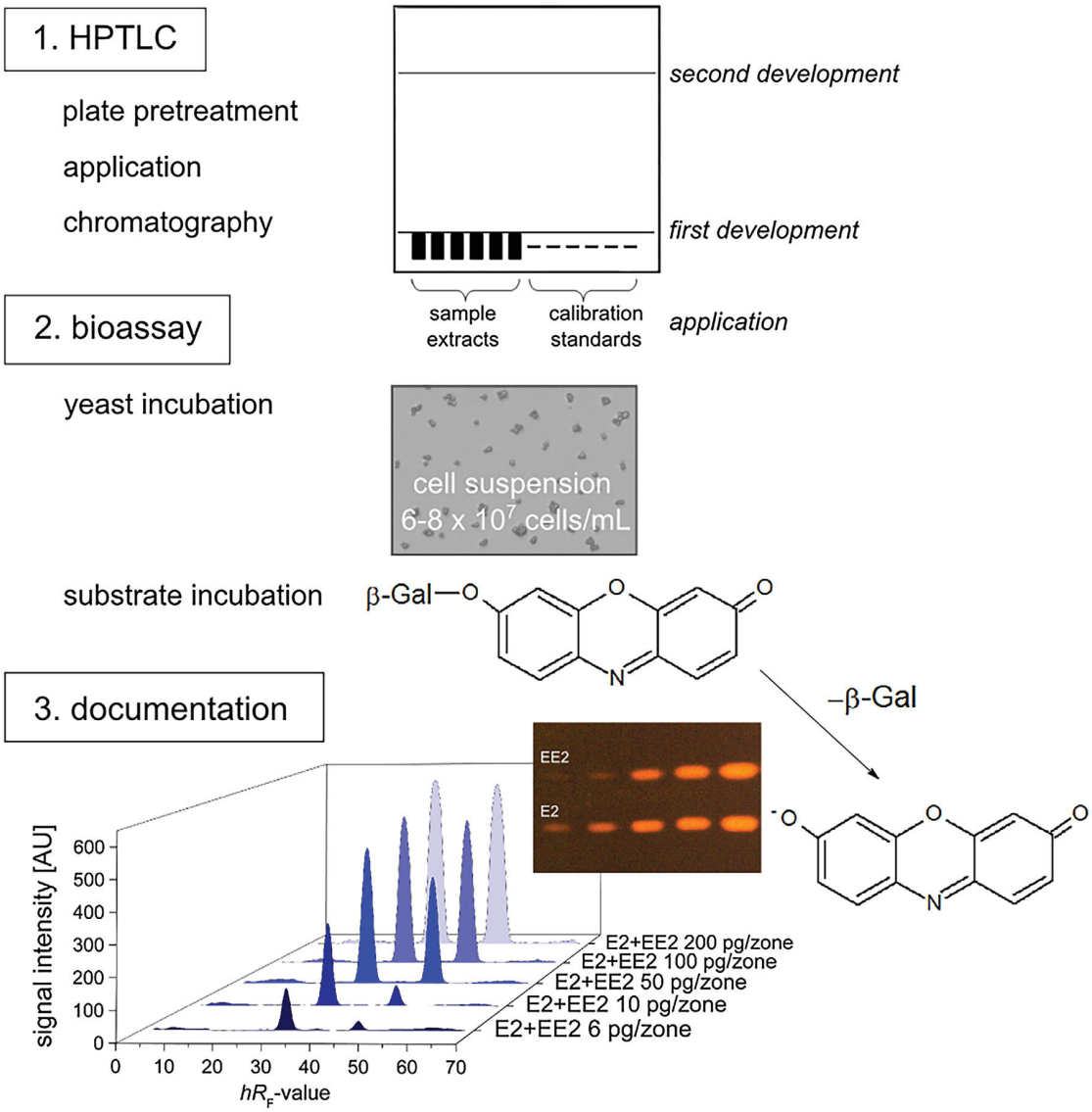

Fig. 1. Schema of the pYES procedure.

Since resorufin is not only a fluorescing but also a chromogenic substrate, the signals were also visible with the naked eye as pink zones on a yellow background, which additionally can be detected in absorption mode at 572 nm, but with a loss of sensitivity.

3.5. Limits of detection and quantitation and working range

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by means of the signal-noise-ratio (S/N). LOD is defined as S/N ≥ 3 and LOQ as S/N ≥ 10. On average, the LOD for E2 and EE2 was determined to 3 ± 0.5 and 4 ± 0.7 pg/zone, respectively; the LOQ was 5 ± 1.8 and 8 ± 1.0 pg/zone, respectively (n = 7).

The suitable range for the quantitation for E2 and EE2 was chosen on the basis of the determined LOD and the correlation of calibration curves of several measurements. A good correlation was obtained in a range of LOD-200 pg per zone for both E2 and EE2 (Fig. S6). The coefficients of determination (R^2) were between 0.9538 and 0.9911 for E2 and between 0.9927 and 0.9992 for EE2 (n = 9). Especially at low amounts of E2 and EE2, the relative standard deviations (% RSD) of the obtained signal were relatively high, which is due to the fact that the tests outcome is dependent on i) the daily condition of the yeast cells, ii) the number of cells and iii) the background expression of β-galactosidase. On average, the RSD of the individual measuring points of the calibration graph (Fig. S6) was 25% RSD for both E2 and EE2.

3.6. Sample extraction and recovery rates

To show the applicability of the utilized liquid–liquid extraction (LLE) over the previously defined working range, recoveries of E2 and EE2 at four different levels from aqueous solutions were determined. Therefore, ultrapure water was spiked with E2 and EE2 in concentrations of about 2, 6, 14, and 20 ng/L, and aliquots of 30 mL were extracted with TBME. After evaporation of the TBME, the residue was dissolved in 200 μL of ethanol, resulting in a 150-fold enrichment. Application of 50 μL of the extracts onto an HPTLC plate resulted in amounts of about 15, 45, 105, and 150 pg/zone for E2 and EE2, which were well in the calibration range. The obtained

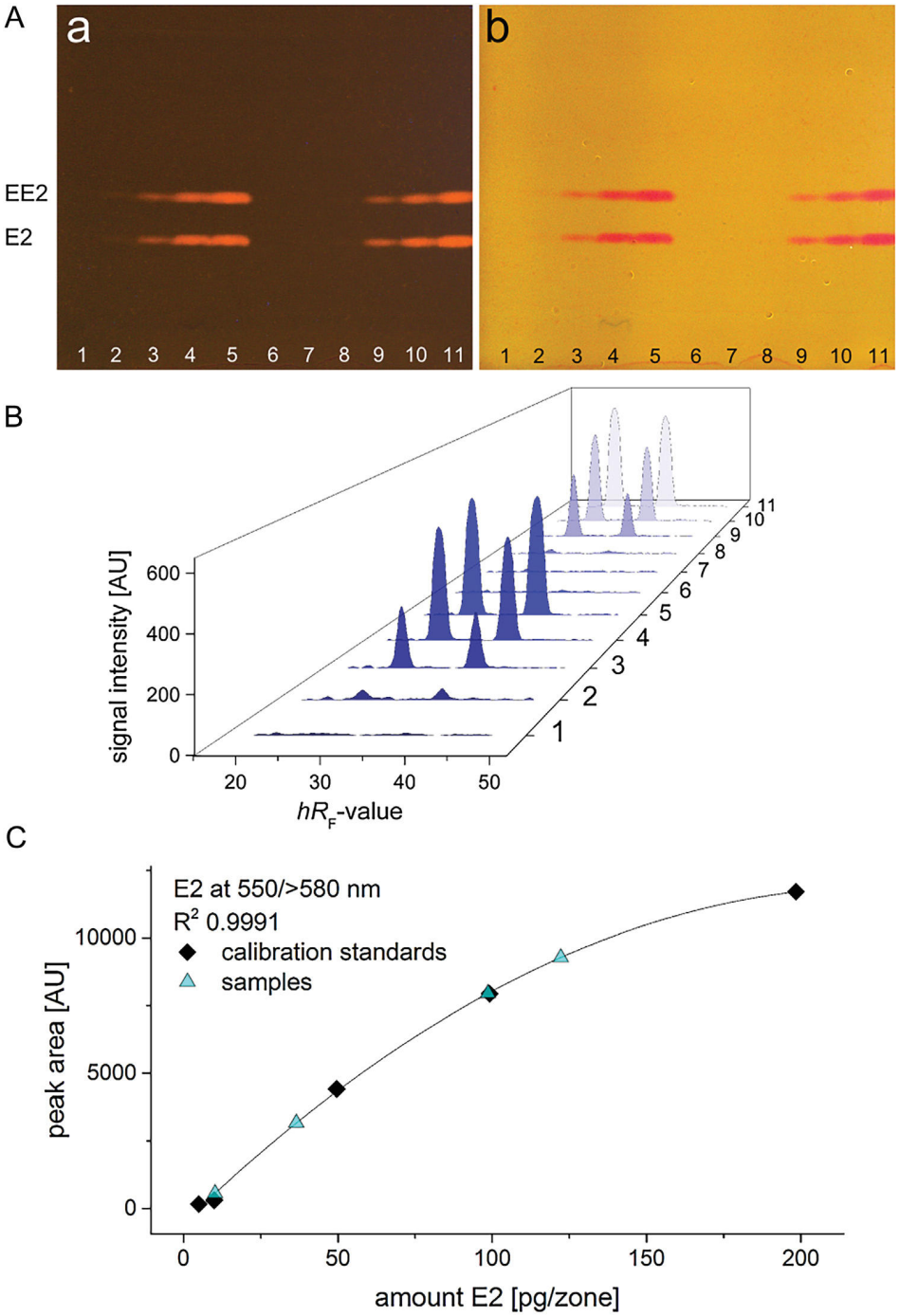

Fig. 2. (A) pYES of a blank sample extract (track 1), spiked and extracted water samples (tracks 2–5: extracts 2 ng/L, 6 ng/L, 14 ng/L, 20 ng/L), and calibration standards of E2 and EE2 (tracks 6–11: 3, 5, 10, 50, 100, 200 pg/zone) on HPTLC RP–18 W plates under UV 254 nm (a) and under white light illumination (b); (B) corresponding 3D densitogram of the fluorescence scan at 550/ > 580 nm; (C) calibration curve exemplarily for E2 at 550/ > 580 nm, analyzed samples are displayed as a triangle.

Table 2
Recoveries for E2 and EE2 from spiked water samples (n = 6).

Substance	Spiked level [ng/L]	Determined concentration [ng/L]	Recovery [%]	RSD [%]
E2	1.98	1.80	91	15
	5.95	6.45	108	25
	13.89	14.26	103	17
	19.84	19.55	99	17
EE2	1.99	2.09	105	16
	5.98	6.21	104	20
	13.94	14.83	106	15
	19.92	22.50	113	17

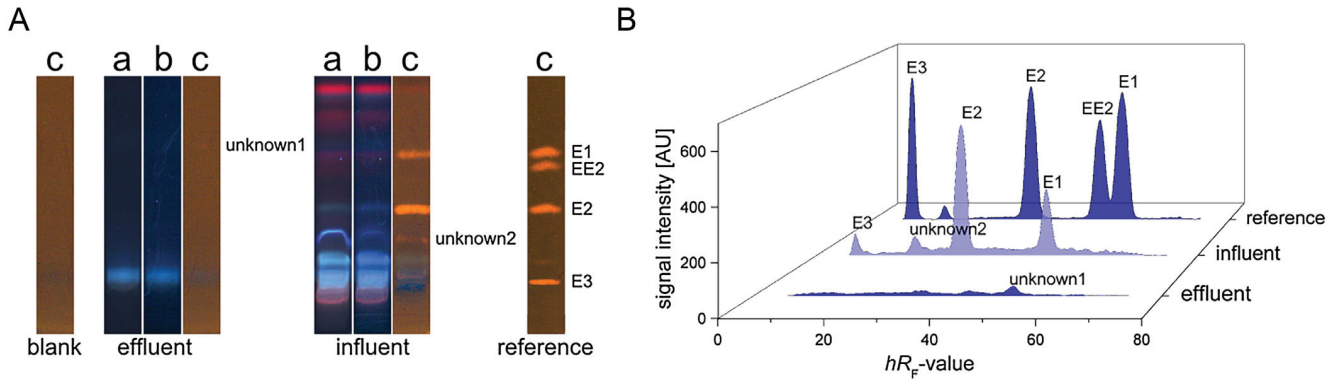

Fig. 3. (A) Tracks of a blank sample extract, extracted waste water samples (effluent and influent), and a reference track with E3 5 ng/zone, E2 100 pg/zone, EE2 100 pg/zone and E1 1 ng/zone after chromatography under UV 366 nm (a), after yeast incubation under UV 366 nm (b), and after pYES under UV 254 nm illumination with orange zones showing estrogenic activity (c); (B) 3D densitogram of a fluorescence scan at 550/ > 580 nm of the tracks c after pYES. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of pYES for original and hydrolyzed effluent and influent waste water samples; observed increase of the peak areas after hydrolysis written in bold.

substance	hR_F	original effluent	hydrolyzed effluent	%peak area hydrolyzed/original effluent	original influent	hydrolyzed influent	%peak area hydrolyzed/original influent (n = 4)
E3	9	–	–	–	+[a]	+[a]	111 ± 12% RSD
unknown2	23	–	–	–	+	+	**195** ± 7% RSD
E2	34	–	–	–	16.9 ng/L (± 11% RSD, n = 4)	17.0 ng/L (± 18% RSD, n = 4)	99 ± 7% RSD
EE2	46	–	–	–	–	–	–
E1	51	–	–	–	+[a]	+[a]	**126** ± 9% RSD
unknown1	56	+	+	92 ± 9% RSD	–	–	–

[a] not quantified.

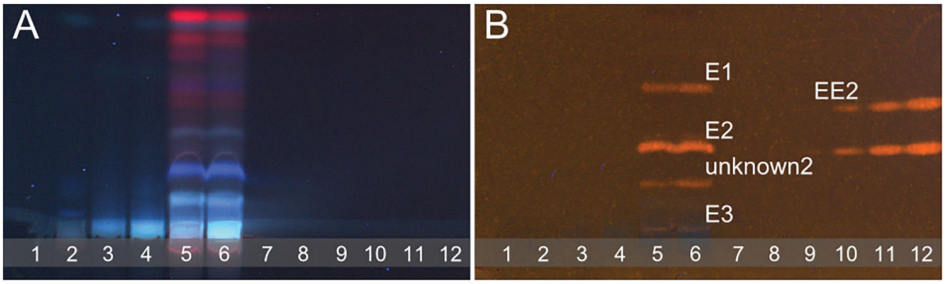

Fig. 4. Plate images after pYES of extracts of original and hydrolyzed waste water samples under UV 366 nm illumination after chromatography (A) and under UV 254 nm illumination after pYES (B); 1 blank (extracted ultrapure water), 2 hydrolyzed blank, 3 original effluent, 4 hydrolyzed effluent, 5 original influent, 6 hydrolyzed influent, 7–12 calibration standards: 3, 5, 10, 50, 100, 200 pg/zone of E2 and EE2, respectively.

results are exemplarily shown in Fig. 2. The recovery experiments were performed on six days and revealed mean recoveries of 100% and 107% for E2 and EE2, respectively (Table 2). The high recoveries demonstrate that the developed screening (extraction plus pYES) showed a good accuracy. Relative standard deviations up to 25% are appropriate, considering that the method works with microorganisms in a low nanogram range. With a sample volume of 30 mL, a concentration factor of 150 and an application volume of 50 μL, the developed method is suited for the detection of E2 and EE2 in samples down to 0.4 ng/L for E2 and 0.6 ng/L for EE2. Hence, the developed pYES meets the requirements of the maximal acceptable method detection limit of 0.4 ng/L for E2 of the EU watch list [1]. However, to meet the maximal acceptable method detection limit of 0.035 ng/L for EE2, the extraction of higher sample volumes is required.

3.7. Analysis of waste water samples

Finally, the analytical procedure was tested with real samples, influent and effluent waste water of a local waste water treatment plant. Under UV 366 nm illumination, especially the influent extracts showed several zones with blue and red fluorescence after chromatography and still after the yeast incubation (Fig. 3 A). However, after substrate incubation, some of these zones were weaker or not visible anymore, whereas zones with estrogenic activity became visible by an orange fluorescence, and no interferences were detected on tracks of blank extracts. Due to the use of the substrate RGP and appropriate settings for densitometry, the scans only showed peaks of zones with an orange fluorescence, thus zones of substances with estrogenic activity, whereas zones with native blue and red fluorescence were suppressed (Fig. 3B). The results of

the pYES of the effluent and influent extracts are summarized in Table 3. In the influent waste water, E2 was clearly be quantified to about 17 ng/L, EE2 was not detectable, but E3 and E1 could clearly be identified (calibration was not performed), while the effluent waste water was free of them. Besides, two unknown EAC were found in the influent and the effluent (unknown1 hR_F 56, unknown2 hR_F 23).

In a report of the Global Water Research Coalition relative standard deviations of several methods for the determination of EAC in spiked and environmental water samples were presented [23]. The results for the RSD of different bioassays (inter alia two YES assays and an E-Screen) and chromatographic methods were compared. Both YES assays showed about 40% RSD, other bioassays showed at least 25% RSD or even higher RSD. Compared with the RSD presented in the mentioned report, the pYES developed in the present study showed a good precision of 11% RSD (n = 4) for the determination of E2 in the influent samples.

Since hormones are excreted as conjugates (sulfates, glucuronides) [13], the waste water samples were additionally treated with a suitable enzyme to hydrolyze possibly present EAC conjugates. The efficiency of the used glucuronidase was first tested by treating a pure water sample spiked with β-estradiol-17-(β-D-glucuronide) (20 ng/L), and it was observed that 10 μL of the 10-fold diluted enzyme solution were sufficient to hydrolyze the spiked glucuronide (data not shown). Therefore, 30 mL of the waste water samples were treated with the enzyme followed by extraction in the same way as the original samples. Additionally, sample blanks (influent and effluent) without adding enzyme solution were incubated like the enzyme assays, to ensure that the conditions do not cause changes to the sample itself (due to increased temperature and storage time [13]); but the extracts of the sample blanks did not show any differences as compared to the original untreated samples (data not shown). Besides, the enzyme solution was added to ultrapure water and incubated overnight, which verified that the enzyme did not yield false positive signals. After pYES (plate images exemplarily shown in Fig. 4), the fluorescence signal of the unknown EAC at hR_F 23 (unknown2) in the hydrolyzed influent was nearly twice as compared to the original influent (Table 3). A slight increase of the E1 signal was also observed after enzyme treatment. Concerning the other EAC, however, no essential differences between original and hydrolyzed samples were observed.

4. Conclusions

Resorufin-β-D-galactopyranoside was shown to be a rather suitable substrate, releasing orange fluorescing resorufin and allowing the pYES of environmental samples to be performed without interferences by natively blue or red fluorescent contaminants. The modified pYES showed a high selectivity for the targeted analytes since clearly resulting signals only were based on the yeast activity and the produced enzyme, respectively, due to present estrogen active compounds. Due to the minimized sample preparation (LLE, concentration factor of 150) no further steps of purification such as a solid-phase extraction were required, when estrogen active compounds in water samples were extracted with high recoveries and could be quantified down to a low ng/L level. Besides, the presence of conjugated estrogen active compounds in waste water influent samples was proven.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.03.047.

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Supplementary data for

Planar yeast estrogen screen with resorufin-β-D-galactopyranoside as substrate

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Supplementary data

Figure S1. Fluorescence signal (550/>580 nm) of released resorufin depending on the time of substrate (0.1 mg RGP/mL) incubation after application of 1 µg β-galactosidase (n = 3).

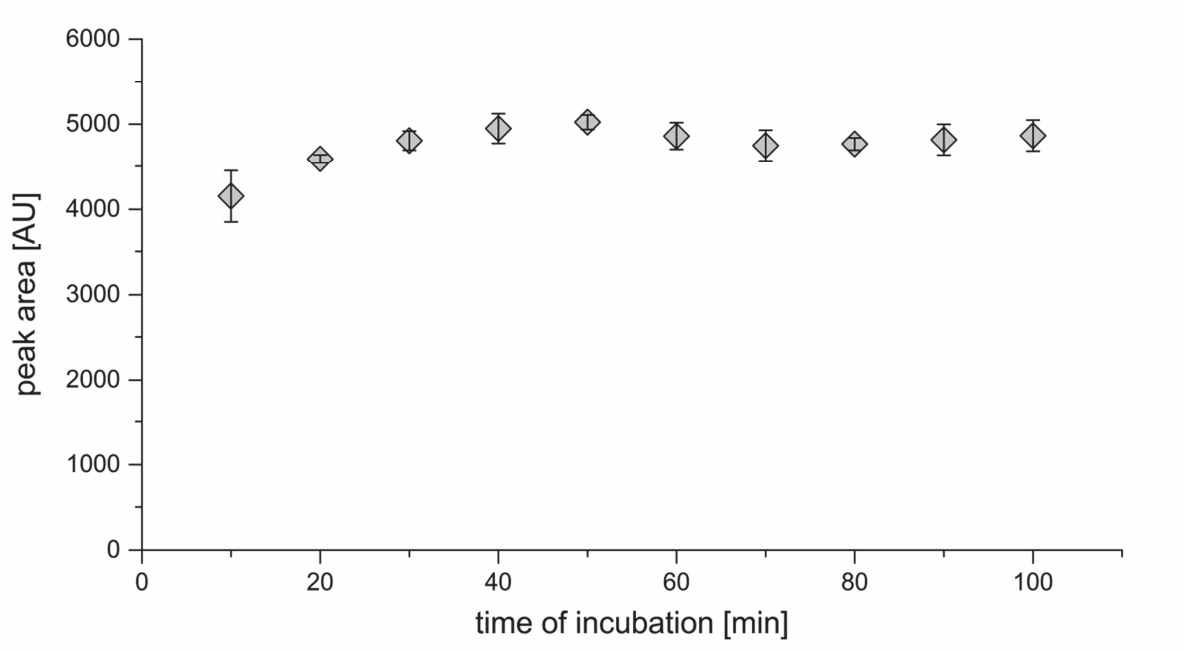
Figure S2. Fluorescence signal (550/>580 nm) of released resorufin depending on the substrate concentration (n = 3, n = 1 for 0.01 and 0.02 mg RGP/mL) after application of 1 µg β-galactosidase.

Figure S3. Fluorescence signal (550/>580 nm) of released resorufin after application of 10 pg E2, incubation (3 h) with yeast cells at different densities (up to 5.5 x 10^7 cells/mL), dipping into substrate (0.1 mg RGP/mL) and incubation (37℃ for 30 min).

Figure S4. Fluorescence signal (550/>580 nm) of released resorufin after application of 10 pg E2, incubation (3 h) with yeast cells at different densities (up to 1.5 x 10^8 cells/mL), dipping into substrate (0.1 mg RGP/mL) and incubation (37℃ for 30 min).

Figure S5. Relative fluorescence signal (550/>580 nm) of released resorufin after application of 10 pg E2 depending on incubation time with yeast cells (n = 4); after 6 h set to 100%.

Figure S6. Calibration graphs for E2 and EE2; fluorescence signal at 550/>580 nm (n = 9).



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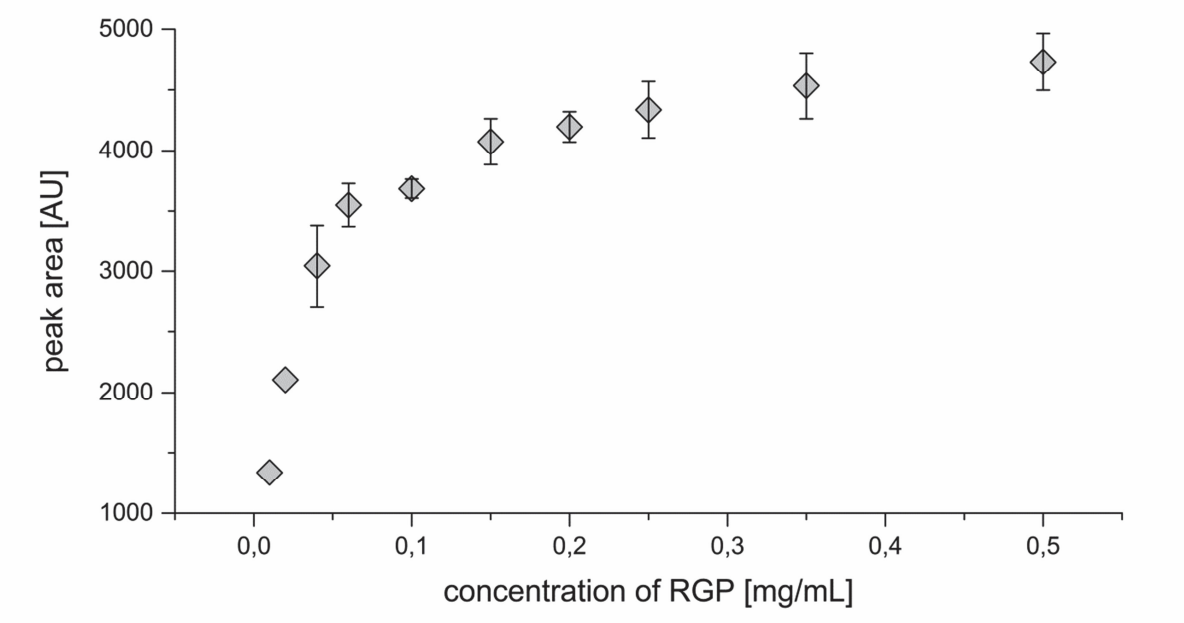

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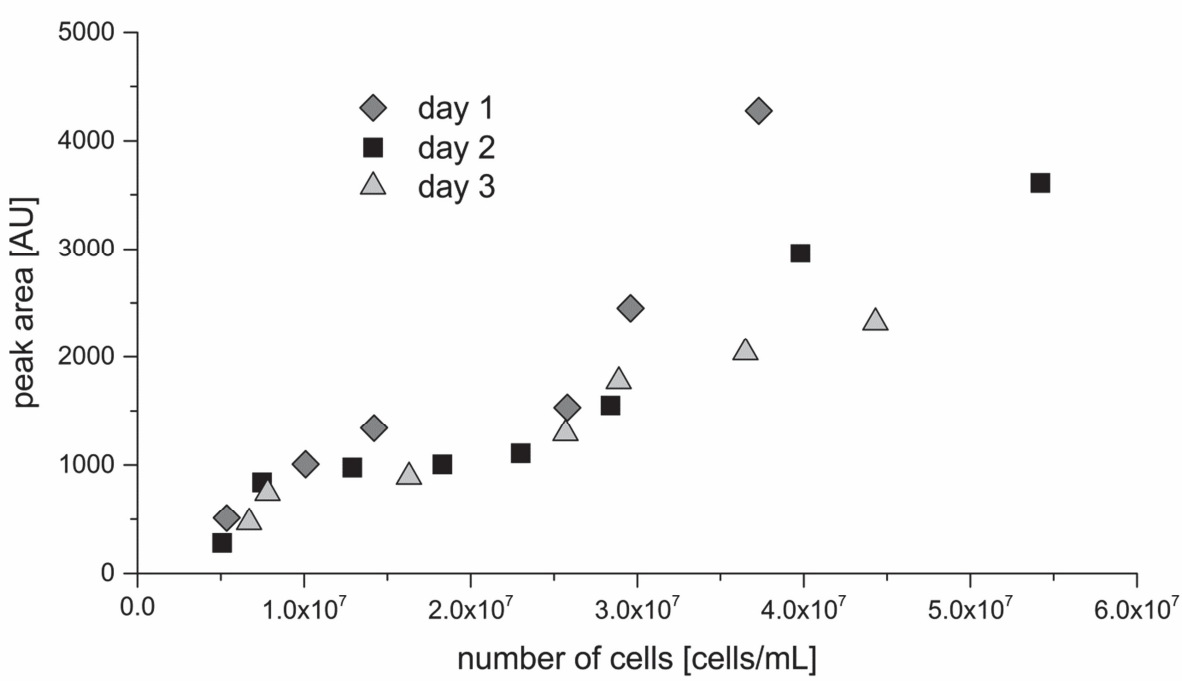

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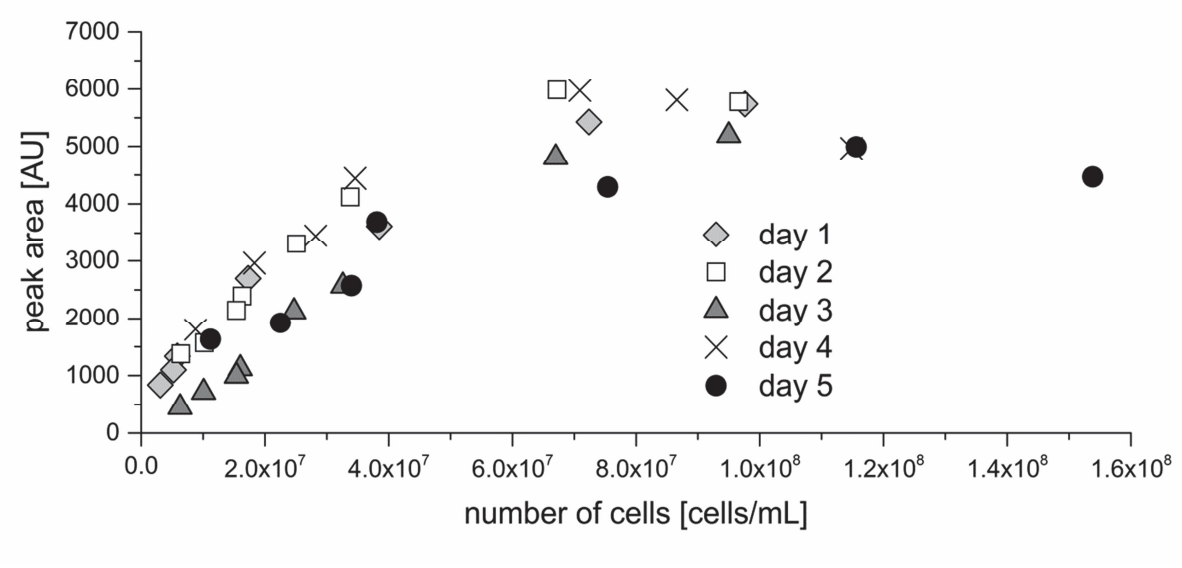

Figure S4. Fluorescence signal (550/>580 nm) of released resorufin after application of 10 pg E2, incubation (3 h) with yeast cells at different densities (up to 1.5 x 10^8 cells/mL), dipping into substrate (0.1 mg RGP/mL) and incubation (37℃ for 30 min).

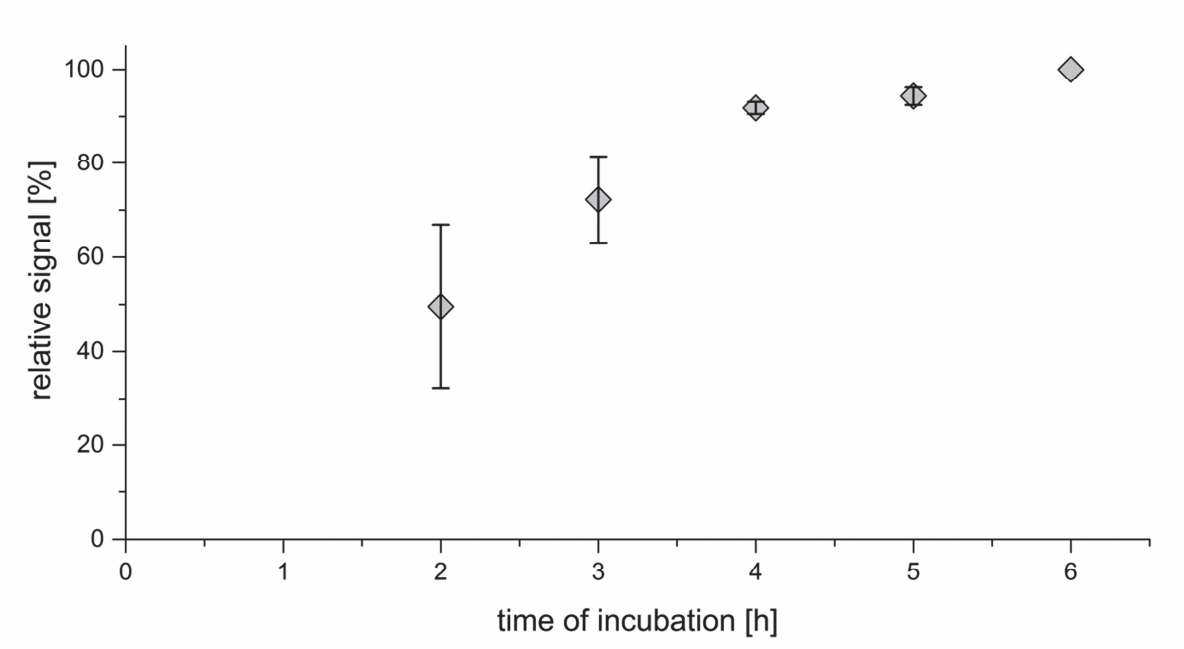

Figure S5. Relative fluorescence signal (550/>580 nm) of released resorufin after application of 10 pg E2 depending on incubation time with yeast cells (n = 4); after 6 h set to 100%.

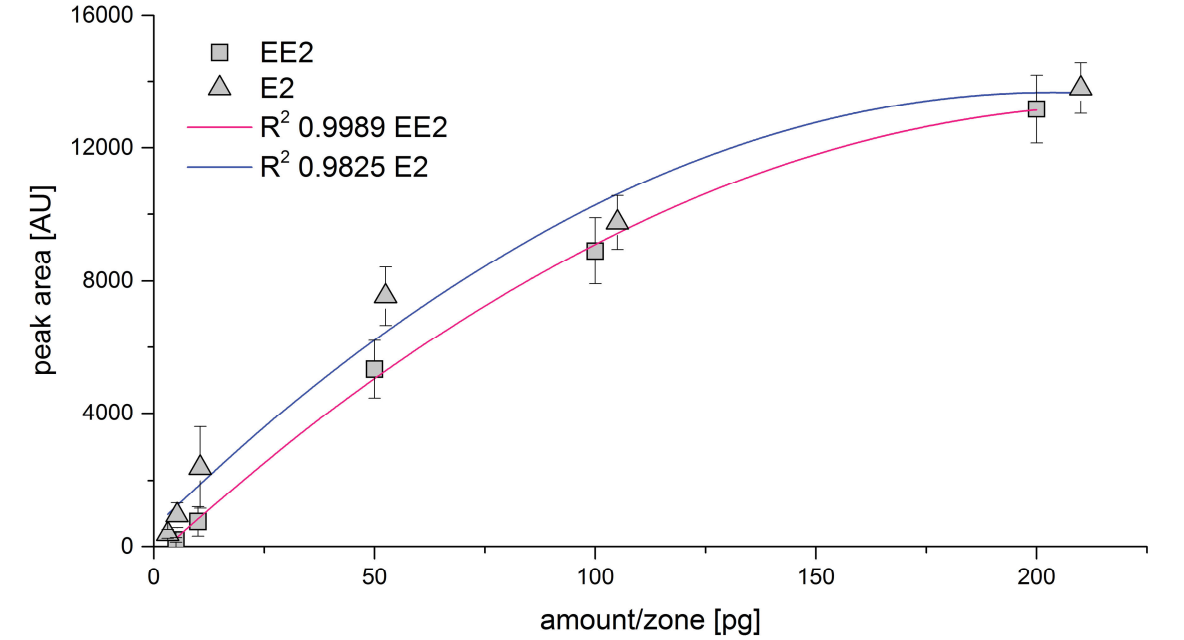

Figure S6. Calibration graphs for E2 and EE2; fluorescence signal at 550/>580 nm (n = 9).

2 Logit-log evaluation of planar yeast estrogen screens

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Logit-log evaluation of planar yeast estrogen screens

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ABSTRACT

Receptor assays like the yeast estrogen screen (YES) performed in microtiter plates normally provide dose-response curves with a sigmoidal shape in semi-log plots. Such sigmoidal plots can be linearized by the logit function resulting in logit-log plots, as mainly known for the evaluation of enzyme-linked immunosorbent assays and radioimmunoassays. Since the planar yeast estrogen screen (pYES) represents the transfer of the receptor assay YES to high-performance thin-layer chromatography (HPTLC), it was assumed to obtain sigmoidal shaped dose-response curves from the measured signals, which subsequently could be used to generate logit-log plots. However, it was observed that typical sigmoidal curves were not obtained, when peak areas were plotted against the applied amount on a logarithmic scale (log amount). Therefore, peak heights were examined in the present study, which revealed proper dose-response curves when plotted against the log amount. The presence of sigmoidal dose-response curves from HPTLC-pYES made it possible to transform the signals into logits and, therefore, to create logit-log plots with linear correlations. The logit-log plots for the estrogen active compounds (EAC) 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) provided a working range up to 500 pg/zone. Applying logit-log plots, mean recovery rates for E2 and EE2 from spiked water samples (2–20 ng/L) were determined to 90% and 108%, respectively, with ≤24% RSD. Moreover, the linear graphs allowed an easy determination of the half maximal effect dose (ED_{50}) of EAC, since the intersection of the graph with the abscissa represents the ED_{50}. Additionally, with the knowledge of the ED_{50} values, the estrogenic potential of EAC in terms of estradiol equivalent factors (EEF) could be determined, resulting in 0.64 for EE2.

1. Introduction

The yeast estrogen screen (YES), introduced by Routledge and Sumpter, is a receptor assay performed in microtiter plates to determine estrogen active compounds (EAC) in terms of effect-directed analysis. For the assay, genetically modified yeast cells containing the human estrogen receptor (hER) and the *lacZ* reporter gene encoding for β-galactosidase were employed [1]. The hER is activated by binding EAC present in a sample, and subsequently the activated hER binds to an estrogen-responsive element causing transcriptions leading to the expression of the reporter gene that results in the production of the enzyme [1]. By enzymatic cleavage, the substrate chlorophenol red β-D-galactopyranoside releases chlorophenol red to be determined by absorbance as indirect signal of the estrogenic activity of EAC [1]. Such ligand-bindings are equilibrium processes that usually show saturation, observable as dose-response curves with a typical sigmoidal shape in semi-log plots. By plotting the YES absorbance signals against the respective concentrations on a logarithmic scale, sigmoidal dose-response curves were obtained, as exemplarily shown by Routledge and Sumpter or van den Belt et al. [1,2].

The transfer of the YES assay to high-performance thin-layer chromatography (HPTLC-planar yeast estrogen screen, HPTLC-pYES) was already shown and provides the great chance to chromatographically separate individual EAC before pYES is performed directly on the HPTLC plate [3–6]. However, when HPTLC-pYES signals expressed as peak areas were plotted against the respectively applied amounts of EAC on a logarithmic scale, typical sigmoidal curves generally were not obtained. Schönborn and Grimmer reported increasing zones and signal intensities, when increasing amounts of EAC were applied, but showed the dose-response relationship for 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) only in a range of 0.5–25 pg/zone, resulting in linear graphs [3]. The dose-response curve for EE2 in a range of 0.3–100 pg/zone was shown by Spira et al., when peak areas were plotted against the applied amount on a log scale, but no real sigmoidal curve was observable [4]. However, by applying a four parametric logistic function curve-fitting model to the data, values for the half maximal effect dose (ED_{50}) were obtained [4]. Results were given in half maximal response instead of half maximal effect

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concentrations (EC_{50}), thus as the ratio of the amounts applied [4]. First, Klingelhöfer and Morlock showed a reliable and profound investigation of E2 equivalency results based on dose-response curves (showing 4 repetitions each) for six different EAC in different ranges using 4-methylumbelliferyl-β-D-galactopyranoside as substrate [5], well-suited also for quantitation of EAC in complex samples [7,8].

The presence of sigmoidal curves with upper and lower asymptotes provides the opportunity to generate logit-log plots as linear calibration curves. The transformation of sigmoidal curves to linear graphs by applying the logit function is mainly used for radioimmunoassays [9–11], and is also known from enzyme-linked immunosorbent assays [12], both performed in cuvettes or microtiter plates. Alternatively, log-log plots or four parameter logistic-log functions were also used [13]. The aim of the present study was to obtain proper dose-response curves from the signals of a recently developed HPTLC-pYES with the substrate resorufin-β-D-galactopyranoside (RGP) [6] and subsequently to apply the logit-log method. The applied HPTLC-pYES utilized the recombinant yeast strain *Saccharomyces cerevisiae* generated by McDonnell et al. [14,15], which is modified with a DNA sequence of the hER and the *lacZ* reporter gene that encodes for β-galactosidase. Enzymatic cleavage of the suitable substrate RGP releasing resorufin indicates estrogenic activity as orange fluorescent HPTLC zones [6]. Since plotting peak areas of the signals of a fluorescence scan against the amount per zone on a log scale did not result in curves with a sigmoidal shape, the peak height as signal of intensity was examined, which indeed showed saturation curves for both E2 and EE2. Hence, it was possible to demonstrate the transfer of the logit-log procedure to HPTLC-pYES, resulting in linear calibration graphs. The applicability of logit-log plots as evaluation tool in HPTLC-pYES was tested with water samples spiked with E2 and EE2, and the obtained results were compared to the results from the former publication [6]. Moreover, the presence of sigmoidal dose-response curves enabled the determination of values for the half maximal effect dose (ED_{50}), on the one hand as the point of inflection of the curve from the sigmoidal curve itself, on the other hand simply from the intersection of the linear graph of a logit-log plot with the abscissa. Thus, it was possible to determine ED_{50} values for E2 and EE2, and, therefore, the estrogenic potential of EE2 in terms of the estradiol equivalent factor (EEF).

2. Materials and methods

2.1. Chemicals and materials

HPTLC glass plates silica gel 60 RP–18 W (200 mm × 100 mm, layer thickness 200 μm), copper(II) sulfate pentahydrate (p.a.), disodium hydrogen phosphate (p.a.), L-histidine (≥99%), hydrochloric acid (37%), potassium chloride (>99%), potassium dihydrogen phosphate (p.a.) and sodium hydroxide solution (20%) were from Merck (Darmstadt, Germany). The following substances for agar plates were from Becton Dickinson (Heidelberg, Germany): yeast nitrogen base without amino acids (YNB), casamino acids [16], adenine and agar. YNB for culture media, ethanol (≥99.8%), methanol (≥99.9%), *t*-butyl methyl ether (TBME, ≥99.8%), 17β-estradiol (E2, ≥98%) and 17α-ethinylestradiol (EE2, ≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium hydrogen carbonate and D-Glucose (anhydrous) were from BDH Prolabo Chemicals (VWR, Bruchsal, Germany). Ethyl acetate (for pesticide residue analysis), *n*-hexane (for pesticide residue analysis), L-lysine (≥98%) and magnesium sulfate heptahydrate (puriss. p.a.) were obtained from Fluka-Sigma-Aldrich (Steinheim, Germany). The substrate resorufin-β-D-galactopyranoside (RGP) was from Santa Cruz biotechnology (Dallas, USA) and dimethyl sulfoxide (DMSO, 99.98%) from Fisher Scientific (Schwerte, Germany). Acetone (≥99.8%) and sodium chloride (≥99%) were from Carl Roth (Karlsruhe, Germany). Ultrapure water was prepared using a Synergy System (Millipore, Schwalbach, Germany). For pYES, yeasts of the strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MATα, PEP4:HIS3, prb1-Δ 1.6R, HIS3-Δ200, lys2-801, trp1-Δ 101, ura3-52gal2can1) were used. The yeast strain was generated by McDonnell et al. [14,15] and the yeasts were stored in a cryo-vial at −70 °C. As optical filter for the TLC Scanner 4 (CAMAG, Muttenz, Switzerland), orange filter glass O 580 from HEBO (Aalen, Germany) was cut to shape.

2.2. Solutions, media and agars

Standard solutions of E2 and EE2 in concentrations of 1 μg/L, 10 μg/L and 100 μg/L were prepared by respectively diluting the stock solutions (1 mg/L). The solvent for standard solutions as well as stock solutions was ethanol. Growth medium was composed as described in [6] and contained 6.8 g/L YNB, 1 g/L glucose, 170 mg/L L-lysine and 100 mg/L L-histidine. Test medium was growth medium plus 112 μM $CuSO_4$. Plate agar was composed of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L) and agar (20 g/L) [6]. A stock solution of RGP (20 mg/mL in DMSO) was diluted in phosphate buffer (40.8 g/L KH_2PO_4, 42.6 g/L Na_2HPO_4, 1.2 g/L $MgSO_4 \cdot 7H_2O$, 3.7 g/L KCl, adjusted to pH 7 with 20% sodium hydroxide solution) to a concentration of 0.1 mg/mL to obtain the substrate dipping solution for pYES [6]. Stock solutions were stored at −20 °C.

2.3. Cultivation of yeast cells for pYES

The cultivation of the yeasts was performed as described in [6]. Briefly, overnight cultures were prepared by inoculation of growth medium and incubation for ≥18 h. Test cultures for pYES with a cell number of 6–8 × 10^7 cells/mL were obtained by centrifugation of the required volume of overnight cultures and re-suspending the cell pellets in test medium.

2.4. Planar yeast estrogen screen (pYES)

The pYES was executed on HPTLC glass plates silica gel 60 RP–18 W prewashed with acetone/water (9/1, v/v). The pH value of the plates was adjusted to about 6.5 with a solution of sodium hydrogen carbonate (25 g/L, pH 6.4) as described in [6]. Sample applications were performed with an Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) on 10 cm × 10 cm HPTLC plates (10 mm from the lower edge, 10 mm from the left side, track distance set to automatic). To examine the relationship between dose and response, different volumes of the standard solutions were applied onto 10 tracks as 5-mm bands resulting in amounts of 3, 4, 5, 6, 10, 50, 100, 200, 500 and 1000 pg per zone for both E2 and EE2. Extracts of water samples (50 μL) were applied as 5 mm × 10 mm areas and standard solutions of E2 and EE2 for calibration as 5-mm bands with amounts of 3, 5, 10, 50, 100, 200 and 500 pg per zone [6]. After application, pYES was performed as described in [6]. Briefly, the separation of the analytes was achieved by chromatography, followed by yeast incubation (6–8 × 10^7 cells/mL, 30 °C, 4 h), substrate incubation (0.1 mg RGP/mL 0.6 M phosphate buffer, pH 7, 37 °C, 3 × 30 min), and documentation of the HPTLC plate. The plates were documented with a TLC Visualizer (CAMAG) under UV 254 nm, UV 366 nm and under white light illumination, and scanned with a TLC Scanner 4 (CAMAG) in fluorescence mode at 550/>580 nm (tungsten lamp). TLC instruments were controlled by the software winCATS, version 1.4.6 (CAMAG).

2.5. Extraction of spiked water samples

As recently described [6], spiked water samples (spiking levels of 2 ng/L, 6 ng/L, 14 ng/L, and 20 ng/L for both E2 and EE2) were extracted with TBME. The separated organic phase was evaporated under a stream of nitrogen, and the residue was dissolved in ethanol. Subsequently, the pYES was performed as described above.

2.6. Curve-fitting and logit-log plot

Sigmoidal curves were obtained by plotting the peak heights of the signals against the decadic logarithm of the applied amount/zone (log amount). For curve-fitting, the software OriginPro (OriginLab, Northampton, USA; version: 2015G Sr2) was used, when the non-linear curve-fitting model for dose-response curves with variable hill slope with four parameters (iteration algorithm for nonlinear regression: Lavenberg-Marquardt, damped least-squares DLS) was applied. The function is described as

$$y = A1 + \frac{A2 - A1}{1 + 10^{(LOGx0 - x)*p}} \quad (1),$$

where *A1* and *A2* are the y values of the lower and upper asymptote, respectively. *LOGx0* gives the point of inflection of the curve ($ED_{50} = 10^{LOGx0}$) and *p* is the hill slope. To form logit values, the peak heights first had to be normalized (*n*) to a range between 0 and 1, employing the bottom and top asymptotes of the sigmoidal curve given out by the software:

$$n = \frac{peak\ \ height\ \ - A1}{A2\ \ - A1} \quad (2).$$

The normalized values were then converted in *logits* by

$$logit = ln\left(\frac{n}{1\ - n}\right) \quad (3).$$

Linear calibration graphs were obtained by plotting the logits of the calibration standards against the respective log amounts. By transforming peak heights of the signals of samples to logit-values, the corresponding log amount could be interpolated from the linear equation:

$$logit = a + b * logamount, \quad (4)$$

where 'amount' is the amount of EAC per zone, *a* the y-intercept and *b* the slope of the line.

2.7. ED_{50} and estradiol equivalents

Values for the half maximal effect dose (ED_{50}) were calculated from the linear equation of logit-log plots since the intersection of the graph with the abscissa (logit = 0) gives the ED_{50}. Additionally, the ED_{50} is an output parameter of the software OriginPro, which determines the value directly from the sigmoidal curves ($ED_{50} = 10^{LOGx0}$).

The estradiol equivalent factor (EEF) for EE2, which represents the estrogenic potential of EE2, was determined as follows:

$$EEF = \frac{ED_{50}\ (E2)}{ED_{50}\ (EE2)} \quad (5).$$

The estradiol equivalent concentration (EEQ) of an unknown in a sample is obtained, when an aqueous sample or a sample extract is applied in different volumes onto the HPTLC plate. The aim is to receive a dose-response curve by plotting the log volumes (μL) against the peak heights. The EEQ is then calculated from:

$$EEQ\ \ \left(pg/\mu L\ \right) = \frac{ED_{50}\ (E2)}{EV_{50}\ (unknown)\ *CF} \quad (6)$$

where ED_{50}(E2) is the half maximal effect dose of E2 (pg/zone), EV_{50}(unknown) the half maximal effect volume of an unknown EAC in a sample, and CF a concentration factor of a possible extraction process.

3. Results and discussion

3.1. Dose-response curves

During the course of HPTLC-pYES, binding of E2 or EE2 results in an activation of the human estrogen receptor integrated in the yeast cells, and the activated receptor binds to a specific DNA sequence, the estrogen-responsive element of the implemented reporter plasmid, causing the transcription of the reporter gene and, thus, the production of β-galactosidase [14,15]. By enzymatic cleavage of the substrate RGP, orange fluorescent resorufin is released, indicating the estrogenic activity of a substance. Plate images and a 3D densitogram of the fluorescence scan of an HPTLC-pYES for E2 and EE2 exemplarily are shown in Fig. 1. To examine the dose-response relationship of E2 and EE2, the obtained peak areas and peak heights were plotted against the amount (pg/zone) and the log amount, respectively, exemplarily shown for EE2 (Fig. 2). Since a ligand-binding reaction usually shows saturation, a typical sigmoidal curve should be observed in a semi-log plot. However, such typical dose-response curves were not obtained when peak areas were used. Instead, the signal steadily increased with an increasing amount per zone, irrespective of the used units (Fig. 2A and C), while simultaneously the zones became wider and wider due to overloading the layer (Fig. 1). It first was supposed that the high cell density was the reason for the obtained dose-response curves. Therefore, the experiments were repeated with a lower number of yeast cells, but the course of the curve did not change (data not shown). The peak area is proportional to the total amount of the substance over the whole zone and, therefore, increases with the widening of the zones. By contrast, the peak height is in a way limited since it only represents the maximum of the signal regardless its width. Hence, the peak heights were plotted against the amount per zone, when the curve indeed showed saturation (Fig. 2B). A typical sigmoidal curve was then successfully obtained, when peak heights were plotted against the decadic logarithm of the amount per zone (Fig. 2D).

3.2. Logit-log plots and working range

The presence of sigmoidal dose-response curves with upper and lower asymptotes enabled linearization of the data by means of the logit-log method. For this purpose, the peak heights first were normalized to a scale from 0 to 1 (Eq. (2)). This required a preceding curve-fitting to determine the lower and upper asymptotes of the sigmoidal curves, which was performed employing the software OriginPro. Subsequently, logits were calculated from the normalized values (Eq. (3)). Fig. 3 shows the sigmoidal curves for E2 and EE2, when the mean peak heights of five standard calibrations were plotted against the corresponding log amounts in a range of 5–500 pg/zone. After transformation of the data into logits, a linear graph (logit-log plot) was obtained (Fig. 3).

The linear logit-log plots for both E2 and EE2 showed good linearity over two decades (5–500 pg/zone). Linear calibration graphs determined on five days with both new HPTLC plates and yeast cell suspensions, resulted in coefficients of correlation (R^2) between 0.9324 and 0.9990 for E2 and between 0.9877 and 1.0000 for EE2, with means of 0.9927 and 0.9997, respectively (Fig. 3). Compared to former results with polynomial regression and a working range up to 200 pg/zone [6], logit-log plots provided an expanded working range up to 500 pg/zone, which is of great advantage for the screening of samples with unknown concentrations of EAC. The extension of the working range with higher and lower calibration standards is

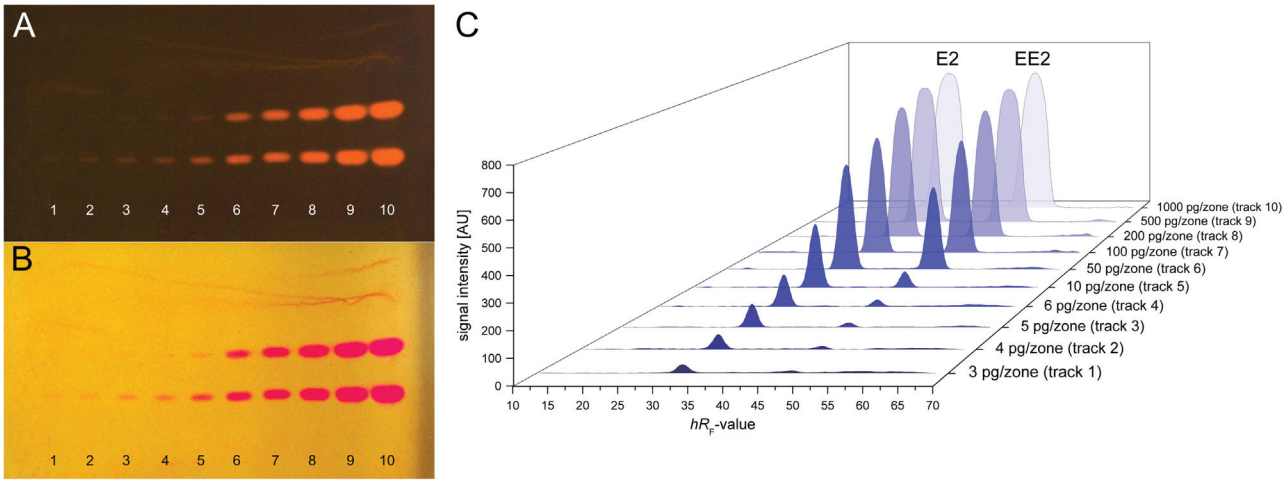

Fig. 1. pYES on HPTLC RP–18 W plates for the determination of the dose-response relationship. Plate images under UV 254 nm (A) and white light illumination (B), and corresponding 3D densitogram (C) of the fluorescence scan (550/ > 580 nm). Enzymatic cleavage of the substrate RGP releases resorufin, detectable as orange fluorescent zones under UV and pink zones under white light. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

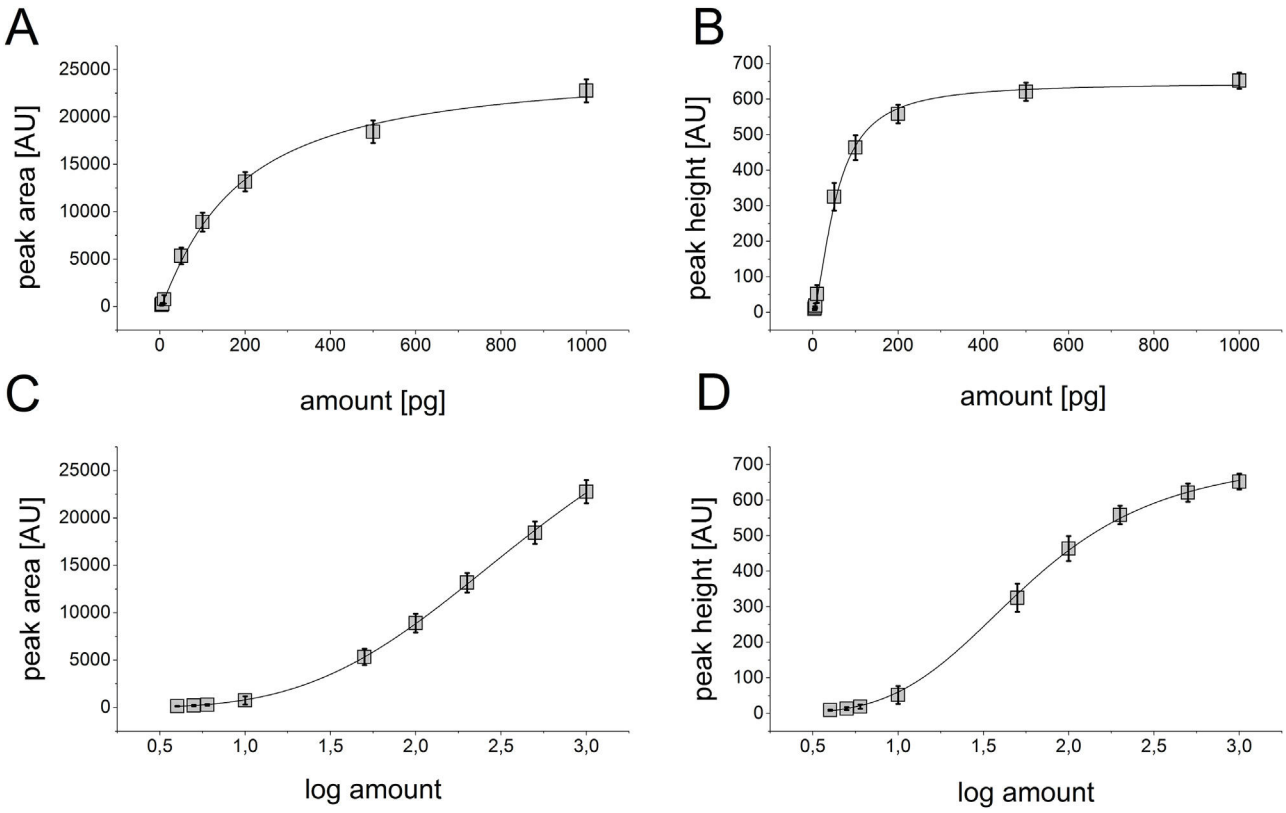

Fig. 2. Dose-response curves exemplarily shown for EE2 (n = 9).

possible as far as a logit-log plot can be formed from the sigmoidal curves. However, it has to be in mind that the highest and lowest calibration standards are limited by the upper and lower asymptotes. Computed values for the upper and lower asymptotes can be lower or higher, respectively, than the highest or lowest standard signals, why logit values cannot be calculated from these signals, thus limiting the working range.

3.3. Using logit-log plots to calculate recovery rates

In an earlier study, water samples were spiked at four different levels (2–20 ng/L), and the extracts were applied on an HPTLC plate resulting in amounts of about 15, 45, 105, and 150 pg/zone for both E2 and EE2 [6]. Subsequently, HPTLC-pYES was performed, and recoveries were determined by a polynomial calibration function (using peak areas) in a working range up to 200 pg/zone for E2 and EE2. In the present study, recoveries for the same spiked water extracts were re-analyzed by means of logit-log plots using the peak height of the signals of calibration standards up to 500 pg/zone. Mean recoveries were determined to 90 ± 6% for E2 and 108 ± 10% for EE2 (Table 1) with mean relative standard deviations (RSD) of 17 ± 5%. Thus, recoveries were not identical but rather similar to the results of the former study (mean recovery rates of 100 ± 6% for E2 and 107 ± 4% for EE2 with mean RSD of 18 ± 3% [6]). It should be

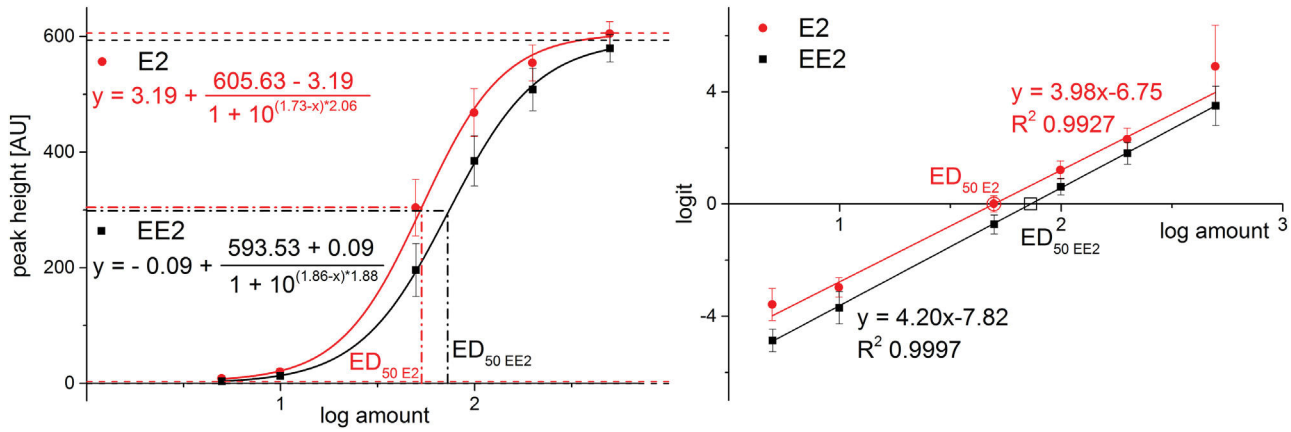

Fig. 3. Dose-response curves with upper and lower asymptotes (n = 5) and respective logit-log plots for E2 and EE2; corresponding ED_{50} is given by the point of inflection of the curve and the intersection with the abscissa, respectively.

Table 1
Recoveries for E2 and EE2 from spiked water samples, calculated by logit-log plots (n = 5).

Substance	Spiked level [ng/L]	Determined concentration [ng/L]	Recovery [%]	RSD [%]
E2	2.0	1.9	97	20
	6.0	5.7	95	19
	13.9	11.8	85	11
	19.8	16.4	83	24
EE2	2.0	2.4	123	19
	6.0	6.1	102	10
	13.9	13.7	98	13
	19.9	21.4	107	22

mentioned that the spiking levels fitted to the lower, nearly linear part of the polynomial calibration curve [6]. Errors expressed as RSD would increase for spiking levels at the upper flattened part of the calibration curve, and *a fortiori* at levels above 200 pg/zone (Fig. 2A). That is the profit of logit-log providing a consistent linear function with a clear slope in the defined working range up to 500 pg/zone.

3.4. ED_{50}, EEF, and EEQ

Sigmoidal dose-response curves also allowed the determination of the half maximal effect dose (ED_{50}) [4] instead of an EC_{50} used in microtiter plate assays and, therefore, the estrogenic potential of an EAC in relation to E2, expressed as estradiol equivalent factors (EEF).

The ED_{50} for E2 and EE2 were determined from the logit-log plots, when the logits of the peak heights of five standard calibrations were plotted against the decadic logarithm of the respective applied amounts per zone. The intersection of the linear graph with the abscissa (logit = 0) represents the ED_{50} of an EAC. Thus, for E2 and EE2, the ED_{50} were calculated to 47 ± 8 and 74 ± 12 pg/zone (n = 5), respectively. By means of ED_{50} values, the estradiol equivalent factor (EEF) of EE2 was determined to 0.64 ± 0.07 (n = 5). In literature, the EEF for EE2 determined by YES according to [1] was reported as 0.9 (0.44-2.1, n = 3) [2]. EEF values for EE2 in other pYES studies were reported to 0.3 [5] and 0.44 [17]. Additionally, the ED_{50} was determined by the software OriginPro from the sigmoidal curves, when again the peak heights of five standard calibrations were plotted against the respective log amounts. As the result, ED_{50} of 50 ± 8 and 74 ± 11 pg/zone (n = 5) were determined for E2 and EE2, respectively, well correlating to the values obtained from the logit-log function. The determined EEF for EE2 was 0.68 ± 0.03 (n = 5) and, thus, was also well comparable to the value determined from the logit-log plot.

In microtiter plate assays, dose-response curves moreover are used for the determination of estradiol equivalent concentrations (EEQ), which are defined as the ratio of the EC_{50} of the reference E2 and the EC_{50} determined from the dilution dose-response curve of a sample, when different sample volumes are applied to the microtiter plate assay, considering any enrichment or dilution factors. The so-called EC_{50} method provides a standardized method for the analysis of bioassays [18]. To estimate the EEQ, the concentration at EC_{50} of the reference E2 is divided by the product of the dilution at the EC_{50} of the sample and the enrichment factor of a possible extraction [18]. This method should also be transferable to HPTLC-pYES. Therefore, the sample has to be applied in different volumes, and the log volumes are plotted against the corresponding signals to obtain a dose-response curve for an unknown EAC. Subsequently, the half maximal effect volume (EV_{50}) is determined from the sigmoidal or logit-log curve. Dividing the ED_{50}(E2) by the EV_{50}(unknown), the EEQ is obtained expressed as pg/μL sample or sample extract (Eq. (6)). Thus, for HPTLC-pYES, the EEQ allows to estimate the estrogenic power of a distinct (separated) unknown EAC, while for YES assays in microtiter plates, the EEQ only represents the total estrogenic activity of a sample.

4. Conclusions

Proper sigmoidal dose-response curves from HPTLC-pYES were obtained after plotting the peak height of resorufin fluorescence signals against the decadic logarithm of the applied amount of 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), respectively. The obtained sigmoidal dose-response curves for E2 and EE2 were successfully transformed into linear logit-log plots, enabling the determination of E2 and EE2 up to 500 pg/zone. This provides a

wide working range, which is greatly advantageous for the analysis of samples with unknown concentrations of estrogen active compounds (EAC). Logit-log plots with linear graphs allow an easy way of interpolation and also the easy determination of the half maximal effect dose (ED_{50}). With knowledge of ED_{50} values for EAC, the determination of estradiol equivalent factors (EEF) of pure compounds and the estradiol equivalent concentration (EEQ) of unknown EAC is possible by HPTLC-pYES.

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3 Detection of estrogen active compounds in hops by planar yeast estrogen screen

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Detection of estrogen active compounds in hops by planar yeast estrogen screen

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ABSTRACT

Hops used in the brewing process of beer for flavoring are known to contain estrogen active compounds (EAC) and to be the source of EAC in beer. The recently developed planar yeast estrogen screen (pYES) with the substrate resorufin-β-D-galactopyranoside (RGP) successfully was applied for the detection of EAC in ethanolic extracts of hops pellet samples. The only pYES positive compound was identified as the hop flavanone prenylnaringenin (PN) by thin-layer chromatography–mass spectrometry. The heat-induced formation of estrogen active PN from the inactive hop flavonoid desmethylxanthohumol was confirmed by simulation of wort boiling, extraction of both the hops' remainder and the supernatant water, and subsequent investigation of the extracts by pYES. By means of the dose-response curve of PN of a hops' remainder extract, the estradiol equivalent concentration (EEQ) and thus the estradiol equivalent amount (EEA) of PN in the hops' remainder after simulation of the wort boiling was determined to 39 μg/L and 52 μg/kg, respectively.

1. Introduction

The presence of estrogen active compounds (EAC) in hops is known for years and impacts on humans were observed as, for example, female hops pickers suffered from menstrual disturbances [1]. Milligan et al. identified four hop flavonoids as EAC, the prenylflavanones 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN) and 6,8-diprenylnaringenin, and the geranylated flavanone 8-geranylnaringenin, when 8-PN was identified as the most potent phytoestrogen [2,3]. The major hop flavonoid is xanthohumol (XN), while other flavonoids only occur in trace amounts [4], but the formation of isoxanthohumol (IXN) from XN and the estrogen active compounds 6-PN and 8-PN from desmethylxanthohumol (DMX) – inter alia during the brewing process of beer – was reported [5,6]. Thus, more prenylflavanones (partly with estrogenic activity) than prenylchalcones occur in beer [7].

In the present work, hops pellet samples were extracted with ethanol, and the extracts were investigated by planar yeast estrogen screen (pYES) to detect EAC [8]. Additionally, a part of the brewing process was simulated to verify the possible formation of EAC during heat supply in aqueous solutions. Both the hops' remainder and the aqueous phase were extracted, analyzed by pYES, and the results compared to them obtained with the original hops samples. The applied pYES provided the separation of the samples by high-performance thin-layer chromatography (HPTLC) prior to the bio-detection by yeast cells of a recombinant yeast strain of *S. cerevisiae* generated by McDonnell et al. [9,10], containing the human estrogen receptor and a reporter gene that encodes for β-D-galactosidase. By cleavage of the suitable substrate resorufin-β-D-galactopyranoside (RGP) by the enzyme that is produced in presence of EAC, orange fluorescent resorufin is released in the HPTLC zone allowing the clear detection of an estrogenic activity of a substance [8]. Moreover, detected EAC were identified by HPTLC coupled to electrospray ionization mass spectrometry.

In a recent work, the applicability of the logit-log method as evaluation tool for pYES was demonstrated, allowing the easy determination of half maximal effect doses (ED_{50}) of EAC and the calculation of estradiol equivalent factors (EEF), using the example of 17α-ethinylestradiol (EE2), a synthetic estrogen used in contraceptives [11]. Additionally, the use of pYES for the estimation of estradiol equivalent concentrations (EEQ) of unknown EAC in liquid samples or sample extracts with the EC_{50} method was taken into account [11]. These calculations were used in the present study to exemplarily estimate the EEQ of hops' remainder extracts and hence the estradiol equivalent amount (EEA) of the hops' remainder.

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2. Materials and methods

2.1. Chemicals and materials

The following chemicals and materials were from Merck (Darmstadt, Germany): potassium chloride (>99%), potassium dihydrogen phosphate (p.a.), copper(II) sulfate pentahydrate (p.a.), sodium hydroxide solution (20%), disodium hydrogen phosphate (p.a.), L-histidine (≥99%), hydrochloric acid (37%), and HPTLC glass plates silica gel 60 RP-18 W (200 mm × 100 mm, layer thickness 200 μm). Yeast nitrogen base without amino acids (YNB), casamino acids [12], adenine and agar for plate agar were from Becton Dickinson (Heidelberg, Germany). YNB without amino acids for liquid culture media, ethanol (≥99.8%), and methanol (≥99.9%) were obtained from Sigma-Aldrich (Steinheim, Germany). D-Glucose (anhydrous) and sodium hydrogen carbonate were purchased from BDH Prolabo Chemicals (VWR, Bruchsal, Germany). Ammonium formate (≥99%), acetonitrile (≥99.9%), diethyl ether (≥99.8%, ~2% ethanol as stabilizer), L-lysine (≥98%), and magnesium sulfate heptahydrate (puriss. p.a.) were obtained from Fluka-Sigma-Aldrich (Steinheim, Germany). Resorufin-β-D-galactopyranoside (RGP) was from Santa Cruz biotechnology (Dallas, USA), formic acid (98%) and dimethyl sulfoxide (DMSO, 99.98%) from Fisher Scientific (Schwerte, Germany). Acetone (≥99.8%) was from Carl Roth (Karlsruhe, Germany). Ultrapure water was supplied by a Synergy System (Millipore, Schwalbach, Germany). Yeasts of the strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MATα, PEP4::HIS3, prb1-Δ1.6R, HIS3-Δ200, lys2-801, trp1-Δ101, ura3-52gal2can1) for pYES were generated by McDonnell et al. [9,10] and stored in a cryo-vial at −70 °C. Orange filter glass O 580 from HEBO (Aalen, Germany) was used as optical filter for the TLC Scanner 4 (CAMAG, Muttenz, Switzerland).

2.2. Media, agars and cultivation of the yeasts

Media and agars were composed as described in an earlier publication [8]: growth medium consisted of 6.8 g/L YNB, 1 g/L glucose, 170 mg/L L-lysine and 100 mg/L L-histidine. Test medium additionally contained 112 μM $CuSO_4$. Plate agar was composed of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L) and agar (20 g/L). Cultivation of yeasts was performed as described in [8]. Briefly, for overnight cultures, growth medium was inoculated with a colony from an agar plate and incubated overnight. Test cultures were prepared by centrifuging the required volume of overnight cultures and re-suspending the cell pellets in test medium. The cell number was measured with a TC20 automated cell counter (Bio-Rad Laboratories, Munich, Germany).

2.3. Substrate solution

Substrate dipping solutions with a concentration of 0.1 mg RGP/mL were prepared by diluting 200 μL of a stock solution of RGP (20 mg/mL in DMSO, stored at −20 °C) in 40 mL phosphate buffer consisting of 40.8 g/L KH_2PO_4, 42.6 g/L Na_2HPO_4, 1.2 g/L $MgSO_4 \cdot 7H_2O$, 3.7 g/L KCl (adjusted to pH 7 with 20% sodium hydroxide solution) [8].

2.4. Planar yeast estrogen screen (pYES)

HPTLC glass plates silica gel 60 RP-18 W were prewashed with acetone/water (9/1, v/v), heated for 30 min at 120 °C on the TLC Plate Heater III (CAMAG) and cooled down afterwards. To adjust the pH value of the plates to about 6.5, the plates were pretreated with a solution of sodium hydrogen carbonate (25 g/L, pH 6.4) [8]. Sample extracts were applied as 8 mm × 20 mm areas with an Automatic TLC Sampler 4 (ATS4, CAMAG) onto 10 × 10 cm HPTLC plates (15 mm from the lower edge, 12 mm from the left side, track distance set to automatic). The application zones were focused with acetonitrile up to 25 mm in a twin-trough chamber (10 cm × 10 cm, CAMAG). Chromatography was performed with a mixture of MeOH/H_2O (3/2, v/v) up to a migration distance of 70 mm using an Automatic Developing Chamber 2 (ADC2, CAMAG). Afterwards, yeast incubation (6–8 × 10^7 cells/mL, 4 h, 30 °C) and substrate incubations (RGP in 0.6 M phosphate buffer, 0.1 mg/mL, pH 7, 3 × 30 min, 37 °C) were performed as described in the earlier work [8]. HPTLC plate documentation was performed with a TLC Visualizer and a TLC Scanner 4 (both CAMAG). Plate images were captured under UV 254 nm, UV 366 nm and white light illumination, and the HPTLC plates were scanned in fluorescence mode at 550/ > 580 nm (tungsten lamp). TLC instruments were operated by the software winCATS, version 1.4.6 (CAMAG).

2.5. Samples and extraction

Hops pellet samples were obtained from the Distillery for Research and Training, University of Hohenheim, Germany. Two samples without further information about the hop variety were from Germany (hops A and B), one sample was from Great Britain (variety First Gold) and one from the USA (variety Galena). The hops pellets were ground in a mortar before extraction. The pestled hops sample (0.15 g) was extracted with ethanol (1 mL) by sonication for 10 min, followed by centrifugation at 13,000*g* for 5 min. The supernatant extract was evaporated in a nitrogen stream and the residue dissolved in 200 μL of ethanol. Subsequently, the extract was applied (4 μL) onto an HPTLC plate and subjected to pYES as described above (2.4 pYES). Additionally, the wort boiling part of the brewing process was simulated by cooking the pestled hops (0.15 g) in water (0.1 L) for one hour under constant stirring. In parallel, this process was also performed without heating. After centrifugation, the hops' remainders were extracted with ethanol as described above. The supernatant aqueous extract (1 mL) was extracted with diethyl ether (2 mL) by vortexing for 2 min [13]. The separated organic phase was evaporated by a nitrogen stream, and the residue was dissolved in 200 μL of ethanol. After the application of the extracts (4 μL of the hops' remainder extracts, 60 μL of the ether extracts of the supernatant water), pYES was performed as described above (2.4 pYES).

2.6. High-performance thin-layer chromatography–mass spectrometry

An ethanolic extract of the hops' remainder after boiling was applied onto an HPTLC plate (2-fold application of 4, 6, and 8 μL) that was cut into two sections after chromatography. One section of the plate was used for pYES, whereafter the coordinates of active zones were transferred to the other plate section used for mass spectrometry. Zones of interest (8 μL application) were eluted with the oval elution head of a TLC-MS interface (CAMAG) using methanol/ammonium formate buffer (10 mM, pH 4, 98:2, v/v [13]) and the eluate was transferred online at a flow rate of 0.2 mL/min (pump: Jasco PU-980, Groß-Umstadt, Germany) to a single quadrupole mass spectrometer (G1956B MSD, Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) interface. For negative ionization the following parameters were used: capillary voltage 3.0 kV, drying gas temperature 300 °C, drying gas flow rate 10 L/min, and nebulizer gas pressure 40 psig. Mass spectra in negative scan mode were recorded in a range of *m/z* 150 to *m/z* 450 with a fragmentor voltage of 100 V, gain 2.0, a step size of 0.1 and threshold 100. Data was recorded with the software ChemStation B.02.01 SR2 (Agilent Technologies).

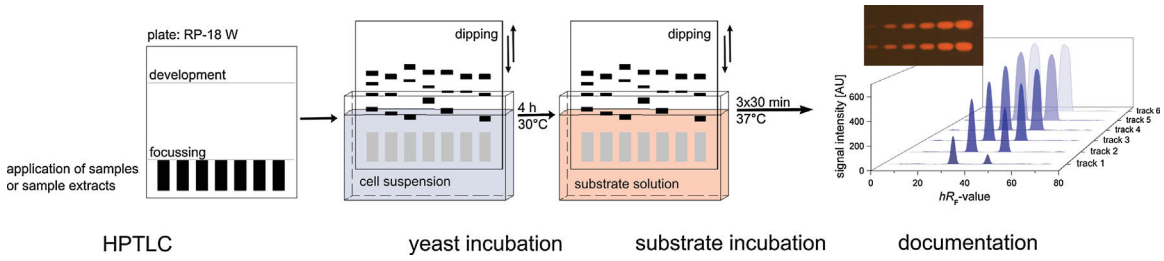

Fig. 1. Work flow of pYES (experimental conditions and details see chapter 2.4).

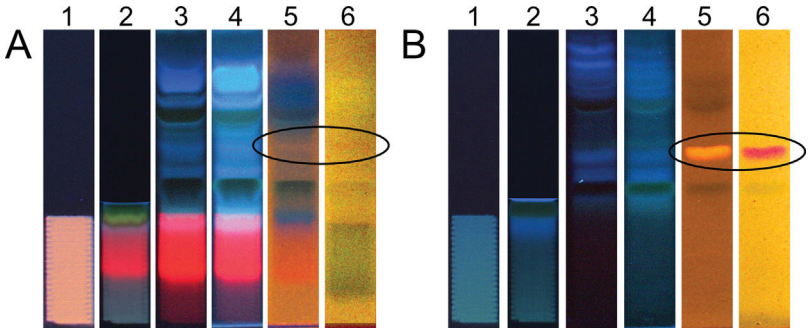

Fig. 2. Plate images of pYES steps for hops extracts (hops B) after direct extraction with ethanol (A) and after extraction of the hops' remainder after simulation of wort boiling (B); 1: after application under 366 nm illumination, 2: after focusing under 366 nm illumination, 3: after chromatography under 366 nm illumination, 4: after yeast incubation under 366 nm illumination, 5: after substrate incubation under 254 nm illumination, 6: after substrate incubation under white light illumination. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

2.7. Half maximal effect volume, estradiol equivalent concentration and estradiol equivalent amount

To estimate the estradiol equivalent amount (EEA) in the hops' remainder after simulation of the brewing process, the ethanolic extract of the hops' remainder (hops B) was 10-fold diluted with ethanol and applied as 8 mm × 20 mm areas in different volumes (2–40 μL) onto an HPTLC plate, followed by pYES (2.4 pYES). The obtained peak heights of the EAC signals were plotted against the decadic logarithms of the respective application volumes. The resulting sigmoidal dose-response curve was fitted and the upper and lower asymptotes of the curve were determined using the software OriginPro (OriginLab, Northampton, USA; version: 2015G Sr2). With knowledge of the lower and upper asymptotes, a logit-log plot for the EAC in the hops' remainder was created as described in an earlier work [11]. From the logit-log plot, the half maximal effect volume (EV_{50}) of the EAC was determined (intersection with the abscissa) and consequently the estradiol equivalent concentration (EEQ) of the hops' remainder extract was calculated as follows:

$$\mathrm{EEQ(pg/\mu L)} = \frac{\mathrm{ED_{50}(E2) * DF}}{\mathrm{EV_{50}(EAC)}}, \quad (1)$$

where $ED_{50}(E2)$ is the half maximal effect dose of E2 (pg/zone) and *DF* is the dilution factor of the extract applied. The EEA of the spent hops was then estimated from:

$$\mathrm{EEA}(\mu\mathrm{g/kg}) = \frac{\mathrm{EEQ}(\mu\mathrm{g/L})}{\mathrm{cont(kg/L)}}, \quad (2)$$

where *cont* represents the content of hops' remainder in the extract.

3. Results and discussion

3.1. Estrogen active compounds in hops

Ground hops pellets were extracted with ethanol and the extract was investigated by pYES (schema of pYES steps see Fig. 1). Milligan et al. identified the four flavanones 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), 8-geranylnaringenin and 6,8-diprenylnaringenin as estrogen active compounds (EAC) in hops [3]. However, only one HPTLC zone with estrogenic activity was observed in the extract of the pestled hops sample as very weak orange fluorescing zone under 254 nm illumination (Fig. 2A, track 5) and very weak pink zone under white light illumination (Fig. 2A, track 6), respectively. Since 80–90% of the flavonoids of hops were determined as xanthohumol (XN), and estrogen active flavonoids only occur in trace amounts [4,14], a low amount of EAC in the hops extract and, thus, only weak signals were expected. Moreover, the estrogenic activity of 8-PN was reported to be considerably higher than the activity of the other detected estrogenic hop flavonoids [2,3], why it was plausible to obtain only one HPTLC zone showing estrogenicity after pYES.

3.2. Direct extraction versus extraction after simulation of wort boiling

Pestled hops pellets were extracted with ethanol and the extracts were compared to extracts of the hops' remainder after the simulation of wort boiling. Plate images of the pYES steps are shown in Fig. 2. In both extracts only one HPTLC zone with estrogenic activity was observed, indicated by orange fluorescence of released resorufin (Fig. 2, tracks 5). However, the orange fluorescing HPTLC zone of the hops extract became much more clear and intensive after heating in water (Fig. 2B, track 5 versus Fig. 2A, track 5). The treatment with water also acted like a pre-extraction cleanup leading to the removal of some matrix compounds, clearly to see, since the hops' remainder extract showed less native fluorescing zones compared to the extract of the original hops sample (Fig. 2B, track 3 versus Fig. 2A, track 3). The formation of prenylflavanones from their chalcones during the brewing process, including the isomerization of inactive desmethylxanthohumol (DMX) to estrogen active 6-PN and 8-PN (Fig. 3), respectively, was reported [5,6,15]. In agreement with the reports, the heat treatment in water led to the formation of a higher amount of EAC detected by pYES, simul-

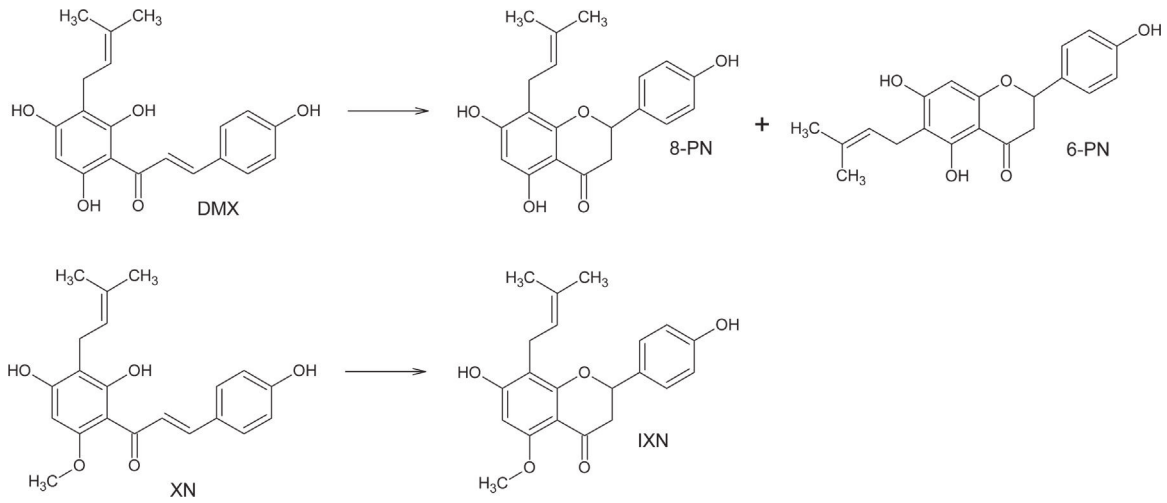

Fig. 3. Isomerization of desmethylxanthohumol (DMX) to 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN), and isomerization of xanthohumol (XN) to isoxanthohumol (IXN).

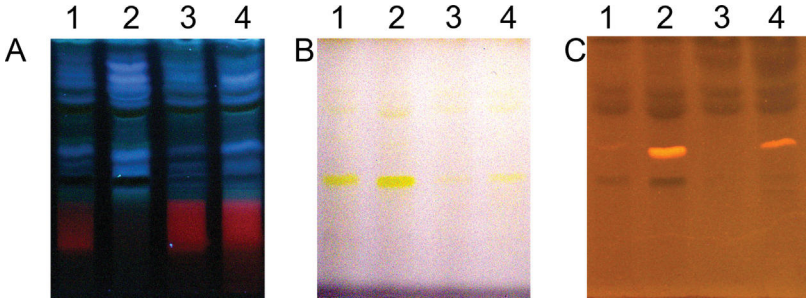

Fig. 4. Plate images of extracts of hops B' remainders and extracts of the supernatant water after chromatography under 366 nm illumination (A), after yeast incubation under white light illumination (B), after substrate incubation under 254 nm illumination (C); extracts of hops' remainders after cold (1) and hot (2) water treatment, extracts of the supernatant water after cold (3) and hot (4) treatment.

taneously showing the advantage of the application of an HPTLC method since different samples can be applied in parallel and differences in the amounts of detected EAC are instantly observable at one look. The experiments show the great advantage of RGP as pYES substrate, when blue fluorescing matrix compounds do not interfere with the detection of EAC, as it is to be respected, if 4-methylumbelliferyl-β-D-galactopyranoside is used [13].

3.3. Impact of hot/cold water

Hops samples were stirred in cold and hot water for one hour, whereafter the hops' remainders were extracted with ethanol. According to Klingelhöfer and Morlock [13] the supernatant water phases were extracted with diethyl ether. As seen in Fig. 4C, the EAC was detectable in the extracts of the hops' remainders of both the cold and hot water treatment, but clearly increased after heat treatment. The EAC was also clearly be detected in the aqueous phase after heat treatment, but the aqueous phase extract of the cold water treatment did not show any zone of estrogenic activity. Thus, a transfer of the EAC into water occurred under heat treatment (comparable to the brewing process), but obviously not into cold water, or the amount of EAC in the cold water extract was too low to be detected.

Stirring in cold water slightly decreased the amount of matrix substances in the hops' remainders extract (Fig. 4A, track 1 versus Fig. 2A, track 3, and Fig. 4C, track 1 versus Fig. 2A, track 5), while additional heating not only increased the aqueous extraction of matrix substances (Fig. 4A, track 2 versus track 1), but also the amount of EAC in the hops' remainder (Fig. 4C, track 2 versus track 1).

The application of an HPTLC method provides the advantage to see all extracted sample components at one look. Hence, differences in the amount of the components can be seen directly on the plate, especially to be seen in the extracts of the hops' remainder under UV 366 nm illumination. The extract of the hops' remainder after the cold water treatment showed a couple of zones of blue and red fluorescing compounds (Fig. 4A, track 1), which clearly changed in composition after the hot water treatment (Fig. 4A, track 2). Especially the large zone of a red fluorescing compound nearly disappeared, which was, among other compounds, recovered from the ether extracts of the aqueous phase (Fig. 4A, track 4). Thus, heating supported the extraction of this compound from the hops during the stirring in water. As already mentioned above, Fig. 4 also supports the clear distinguishability between native fluorescences of sample components and estrogenic activity signals indicated by the orange fluorescing resorufin and the advantage to see differences in amounts of EAC on the plate at once.

3.4. Mass spectrometry

After chromatography of the hops' remainder extract of hops B (after heating in water), three yellow HPTLC zones were observed (Fig. 5A, track 2), one of which showed estrogenic activity after pYES indicated by orange fluorescence (Fig. 5A, track 1). The HPTLC zones were eluted into a mass spectrometer equipped with an electrospray ionization (ESI) interface.

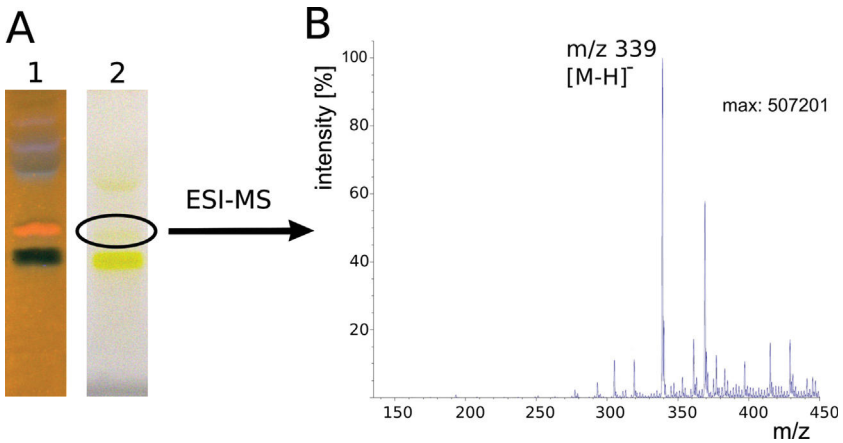

Fig. 5. Images of tracks for HPTLC-MS (A) and corresponding mass spectrum of the HPTLC zone showing estrogenic activity (B); 1: track after pYES under UV 254 nm illumination for transfer of the coordinates of the estrogen active zone, 2: track after chromatography under white light illumination used for MS. The deprotonated molecule at *m/z* 339 confirm the EAC to be prenylnaringenin. Further signals result from the HPTLC plate material and impurities of the used chemicals, respectively. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

In negative ionization mode, the HPTLC zone with estrogenic activity showed a mass signal at *m/z* 339 for the deprotonated molecule, corresponding to the isobaric 6-PN, 8-PN or DMX. Since i) DMX isomerizes completely within 15 min in the brewing kettle [5] and is not known to be estrogen active, ii) 6-PN only showed a weak estrogenic activity (EC_{50} determined by YES according to [16] was a hundred times greater compared to 8-PN [2]), and iii) 8-PN was identified as the most potent phytoestrogen [2], the detected mass of the EAC was assumed to correspond to 8-PN. Besides, Milligan et al. determined the estrogenic activity of 6-PN, 6,8-diprenylnaringenin and 8-geranylnaringenin as very weak and thus stated 8-PN as major compound providing the estrogenicity of hops [3]. However, since there is no clear evidence for the assignment to 8-PN without additional spectroscopic data and DMX can most probably be excluded since it does not show estrogenic activity and would isomerize during heat apply, the EAC is named solely 'PN' hereafter.

The aim of TLC-MS, however, was to confirm the EAC detected by pYES to be the known EAC (8-PN) of hops. Generally, the power of pYES as screening tool was demonstrated, since it was shown a fast and easy non-target screening (no need of standard substances, when up to 20 samples can be analyzed in one run on 20 cm × 10 cm HPTLC plates) to detect EAC in sample extracts after a simple sample extraction without further steps of working up or purification. This was exemplarily shown for ethanolic extracts of hops pellet samples to investigate other samples than water samples (as already shown in [8]). Thus, it was shown, with another matrix, the detection of EAC was also possible and the differentiation between native fluorescence and fluorescence due to estrogenic activity was given. Moreover, the possibility for an identification of unknowns by coupling HPTLC to MS was shown. Additionally, for the first time, the application of the EC_{50}-method in an HPTLC analysis (see chapter 3.6) to estimate the estrogenic potential of a sample or single sample compounds in terms of estradiol equivalents was demonstrated, for which, however, the identity of an EAC does not have to be necessarily known.

Additionally to the EAC, the upper and lower natively yellow zones (Fig. 5A, track 2) were analyzed and showed mass signals at *m/z* 361 ($[M-H]^-$) and *m/z* 353 ($[M-H]^-$), respectively. The signal at *m/z* 353 conforms to the deprotonated molecule of the prenylchalcone XN and the prenylflavanone isoxanthohumol (IXN), respectively, and the mass signal at *m/z* 361 is assumed to be the deprotonated molecule of the alpha acids humulone or adhumulone, which are crucial for the bitter taste of beer [17]. Since not only DMX isomerizes to 6- or 8-PN during heat treatment, but also XN isomerizes to IXN (Fig. 3) [5,15], the zone providing *m/z* 353 is assumed to match IXN. Besides, Stevens et al. identified IXN as the main prenylflavonoid in beer [7], why it is plausible that IXN is also the main prenylflavonoid after cooking of hops in water. Furthermore, the zone with *m/z* 353 (XN/IXN) showed the most intensive yellow HPTLC zone (Fig. 5A, track 2). IXN was reported to be a proestrogen, which can be converted to 8-PN by O-demethylation by specific microbiota in the human intestine [18,19], which is of interest since the consumption of beer thus can lead to a higher exposure with EAC than expected from the analysis of beer [18].

3.5. pYES of different hop varieties

Klingelhöfer et al. already screened different samples of beer, aroma hops and bitter hops (Hallertau hops) for the presence of EAC by means of a pYES using the substrate 4-methylumbelliferyl-β-D-galactopyranoside indicating estrogenic activity by the released blue fluorescing 4-methylumbelliferone [20]. Beer samples were extracted with diethyl ether and pestled hops pellets with a mixture of ethanol/methanol/acetone at 60 °C [20]. After pYES, the beer and hops extracts showed up to 3 zones with estrogenic activity [20].

For the present study, four pestled hops pellets from different hop varieties (2 samples from Germany, one from Great Britain and one from the USA) were cooked in water for 1 h, the hops' remainders were extracted with ethanol, and the extracts were examined by pYES. All four extracts only showed a single estrogen active zone after pYES (at the hR_F of prenylnaringenin), but of different intensity (Fig. 6). Hops B (Germany) and the Galena variety (USA) showed more intensive EAC zones than hops A (Germany)

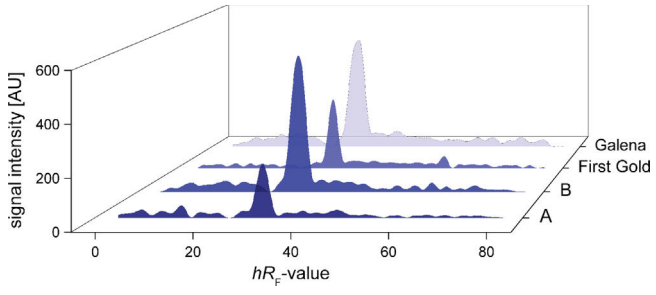

Fig. 6. 3D densitogram of the fluorescence scan at 550/ > 580 nm after pYES of hops' remainder extracts after simulation of the wort boiling of four different hop varieties.

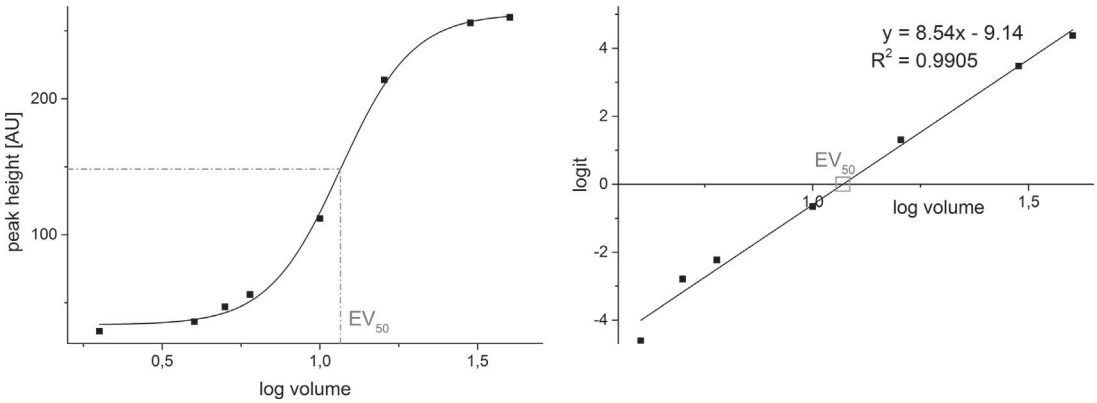

Fig. 7. Dose-response curve and respective logit-log plot for PN in an extract of hops B' remainder after boiling in water; corresponding EV_{50} is given by the point of inflection of the curve and the intersection with the abscissa, respectively.

and the First Gold variety (GB). The differences as compared to the results of Klingelhöfer et al. [20] possibly are due to different pretreatments of hops (cooking in water versus no pretreatment) and both different extractions (cold ethanol, ultrasonic bath versus hot ethanol/methanol/acetone) and chromatographic conditions (mobile phase).

Most, if not all PN in hops extracts and beer is formed by isomerization from DMX (spontaneous cyclization or dependent on storage, medium, heat treatment) [6,14,15]. In the brewing kettle, DMX generally is converted to 6- or 8-PN (Fig. 3), and no DMX or only trace amounts are detectable in beer itself [7]. The abundance of DMX of the total resin flavonoids was determined in 11 different European hop varieties to 2–3% [4]. In another study, DMX was determined in a range of 0.12–0.39% (w/w) in full-grown hop cones of five hop varieties that are used for brewing different ales or bitters [21]. Therefore, the intensity differences of the pYES zones showing estrogenic activity are assumed to be due to the different hop varieties, including different storage times, which are responsible for different amounts of DMX and, thus, for different amounts of PN after boiling.

3.6. *Estimation of the estradiol equivalent amount in the hops' remainder after simulation of the brewing process*

To determine the estradiol equivalent amount (EEA), a hops B sample as example of high EAC hops was submitted to a simulated brewing process, and the hops' remainder was extracted with ethanol. Plotting the pYES signals against the decadic logarithms of different application volumes of the hops' remainder extract, a sigmoidal dose-response curve for the EAC, identified as PN, was obtained (Fig. 7). The presence of a sigmoidal curve allowed the transformation into a logit-log plot and hence the easy determination of the half maximal effect volume (EV_{50}) of the EAC (Fig. 7). The EV_{50} for PN in the hops' remainder extract was determined to 12 μL from the linear logit-log graph. With the EV_{50} value and the dilution factor (DF = 10) and with the half maximal effect dose of 47 pg/zone for E2 [11], the calculation of the estradiol equivalent concentration (EEQ, Eq. (1)) of the hops' remainder extract was calculated to 39 μg/L. Additionally, the estradiol equivalent amount (EEA) of the hops' remainder was estimated (Eq. (2)), resulting in 52 μg/kg, *i.e.* the amount of PN per kg hops after cooking showed an estrogenic activity as compared to 52 μg E2 per kg.

4. Conclusions

The recently developed RGP-pYES was successfully applied on hops samples, and the distinct aspects of the advantageous application of the developed screening based on the chromatographic separation of sample components by HPTLC in combination with a bioassay to detect estrogen active compounds were presented. Thus, estrogen active zones were clearly differentiable from zones of sample constituents with native fluorescence (blue, red), demonstrating the advantageous usage of the substrate RGP. Prenylnaringenin (PN) was identified and confirmed as estrogen active compound after ethanolic extraction of pestled hops pellets and hops' remainders after simulation of the wort boiling, showing the possibility to couple pYES to MS to identify unknown EAC. Additionally, heat induced formation of prenylnaringenin was shown, when the advantage of the application of a planar system became clear since all components of a sample are to be seen at one look on the HPTLC plate and differences in amounts of components are observable at once. Moreover, by creating the dose-response curve and thus the logit-log plot for PN, and by employing the EC_{50}-method (first time use in HPTLC), the possibility to estimate the estradiol equivalent concentrations (EEQ) and the estradiol equivalent amounts (EEA) of single EAC in liquid or solid samples was demonstrated.

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III Discussion

With respect to the watch list of substances for Union-wide monitoring in the field of water policy of the European Commission (2015), which *inter alia* contains four estrogen active compounds (EAC), and with regard to the great number of EAC that occur in our environment, suitable screening methods for estrogenic activity are of great interest. For the determination of EAC, several methods are available. Many of these methods, however, only give a statement about the whole bioactivity of a sample as a sum or represent a targeted analysis of known bioactive substances. The planar yeast estrogen screen (pYES), by contrast, provides the possibility to chromatographically separate single substances of a sample in a planar system and to detect the estrogenic activity of single compounds afterwards. However, samples of interest, especially environmental samples, often contain various native fluorescing compounds (red, blue fluorescence) that can interfere with the detection of EAC when employing the generally used substrate 4-methylumbelliferyl-β-D-galactopyranoside, which releases blue fluorescing 4-methylumbelliferone as positive signal of estrogenic activity. Since screenings are particularly meant to deliver fast results and thus should not need complex sample preparation or purification steps, the pYES represents a suitable method, simultaneously providing a planar clean-up. For this purpose, however, special advancements need to be reached to clearly differentiate pYES fluorescence signals due to estrogenic activity from the native fluorescence of sample compounds.

By usage of the substrate resorufin-β-D-galactopyranoside (RGP), which releases orange fluorescing resorufin as positive signal of estrogenic activity, and by employing fully automated devices, the RGP-pYES was developed as fast screening tool for the detection, determination, and identification of EAC (**chapter II, 1**). Subsequently, the RGP-pYES was successfully applied on sewage and hops pellet samples (**chapter II, 1 and 3**). Moreover, the pYES enabled the determination of estradiol equivalent factors, estradiol equivalent concentrations, and estradiol equivalent amounts of known and unknown EAC in liquid and solid samples (**chapter II, 2 and 3**).

The developed RGP-pYES provided the combination of high-performance thin-layer chromatography (HPTLC) and the detection of EAC by means of genetically modified yeasts in a single test. During method development it became apparent that due to the pH value of the HPTLC plate (water-wettable reversed phase plates, RP-18 W, pH ~4.7) a pretreatment of the plate was necessary (**chapter II, 1**). By treating the plates with a solution of sodium hydrogen carbonate (pH 6.4) the pH of the plate was raised to about 6.5 in order to ensure

an optimal activity of the yeasts and of the enzyme released due to estrogenic activity (**chapter II, 1**). After liquid-liquid extraction (LLE) of water samples with *tert*-butyl methyl ether and enrichment, the extracts were applied as areas whereas the standard solutions were applied as bands onto the pretreated HPTLC plates, followed by pYES (**chapter II, 1**). After chromatography, the plates were dipped in a suspension of recombinant yeasts, containing the human estrogen receptor (hER) and additionally a reporter gen that encodes for the enzyme β-galactosidase. Present EAC are bound by the hER which leads to the expression of the reporter gene and thus to the production of the enzyme. The enzyme cleaves the substrate RGP, which releases orange fluorescing resorufin in the respective HPTLC zones. The developed pYES provided limits of detection of 3 ± 0.5 and 4 ± 0.7 pg/zone for 17β-estradiol and 17α-ethinylestradiol, respectively, (corresponding to 0.4 ng/L and 0.6 ng/L in view of water samples after the employed LLE), and limits of quantitation of 5 ± 1.8 and 8 ± 1.0 pg/zone (n = 7), respectively (**chapter II, 1**). The watch list of the European Commission states maximum acceptable method detection limits of 0.4 ng/L for 17β-estradiol and 0.035 ng/L for 17α-ethinylestradiol. Thus, the developed pYES meets the requirement for 17β-estradiol, but not for 17α-ethinylestradiol. To reach the maximum acceptable detection limit for 17α-ethinylestradiol, however, solely higher sample volumes need to be extracted. The LLE in combination with the pYES provided mean recoveries for spiked water samples at four spiking levels in a range of 2–20 ng/L for both mentioned EAC between 91 and 113% (n = 6) with relative standard deviations (RSD) of 15–25% (**chapter II, 1**). Subsequently, the developed pYES was applied on influent and effluent samples of a waste water treatment plant (plant for about 10,000 inhabitants). Employing pYES for the determination of EAC in these samples, one EAC (unknown) was detected in the effluent, while four EAC (17β-estradiol in a concentration of about 17 ng/L, estrone, estriol and another unknown) were detected in the influent (**chapter II, 1**). Moreover, conjugated EAC were detected in the influent sample (estrone and the unknown EAC), by an additional incubation of the samples with glucuronidase (with an additional sulfatase activity) before extraction and comparison of the pYES-signal intensities (**chapter II, 1**). By the use of RGP as substrate, positive signals of estrogenicity were observable in the form of orange fluorescing zones under UV 254 nm illumination and as pink zones under white light, enabling a clear differentiation from zones of substances with native fluorescences. This impressively shows the enormous advantage of this substrate and the applicability of the RGP-pYES as screening tool for EAC in environmental samples.

The performance of the pYES is based on receptor-transmitted reactions and thus represents a receptor test. At this, respective ligands are bound by the hER whereby the receptor is activated which leads to further reactions. Such processes are usually equilibrium processes that show a saturation behavior, which is observable in sigmoidal dose-response curves, when the obtained signals are plotted against the logarithmized concentrations or amounts, respectively. Saturation curves enable the evaluation of receptor tests by means of the logit-log method performing a transformation of the respective signals into logit-values, whereby linear calibration graphs are obtained as result. The assumption of a possible transfer of this method to pYES was verified and demonstrated (**chapter II, 2**). During examination of the pYES-signals it became apparent that no sigmoidal curves were obtained by using peak areas since an increasing amount of analyte led to wider HPTLC zones and thus to steadily increasing peak areas (**chapter II, 2**). By employing peak heights, in contrast, sigmoidal curves were obtained, which subsequently could be transformed to linear calibration graphs by means of the logit-log method (**chapter II, 2**). Using logit-log plots advantageously allowed the expansion of the working range to 5–500 pg/zone (linear graphs with good correlations over two decades) in comparison to an upper limit of 200 pg/zone when polynomial calibration graphs were used (**chapter II, 1 and 2**). The renewed evaluation of the data obtained for the spiked water samples employing logit-log plots (4 spiking levels in a range of 2 to 20 ng/L, **chapter II, 1**) resulted in mean recoveries for 17β-estradiol and 17α-ethinylestradiol between 83 and 123% (n = 5) with RSD of 10–24% (**chapter II, 2**), and thus showed similarity to the former results (**chapter II, 1**).

The generation of dose-response curves and thus logit-log plots additionally enabled the determination of half maximal effect doses (ED_{50}) for 17β-estradiol and 17α-ethinylestradiol, easily determined from the intersection of the linear calibration graph with the abscissa (**chapter II, 2**). Thus, the estradiol equivalent factor of 17α-ethinylestradiol was determined to 0.64 (**chapter II, 2**). In addition, the theory was enunciated that by employing ED_{50}-values or half maximal effect volumes (EV_{50}) of samples or sample extracts, the EC_{50}-method can be applied for the estimation of estradiol equivalent concentrations (EEQ) and thus the estrogenic potential of samples or sample extracts (**chapter II, 2**). This was demonstrated in the course of the further work (**chapter II, 3**).

To demonstrate the applicability of the developed pYES on further samples, subsequently hops pellet samples were investigated. The presence of EAC in beer – and that hops is the source of these substances – is known for a long time. To show the advantages of the RGP-

pYES, simple hops extracts were examined and compounds showing pYES-positive signals were identified by means of mass spectrometry (MS) afterwards. The extraction of the hops samples was simply executed with ethanol in an ultrasonic bath, and after RGP-pYES, a low amount of an EAC was detected in the extract (**chapter II, 3**). The prenylated flavanone 8-prenylnaringenin (8-PN) is known to be the most potent estrogenic substance in hops. Since during the brewing process of beer the inactive but isobaric prenylated chalcone desmethylxanthohumol is transformed into 8-PN and 6-prenylnaringenin (also a weak EAC of hops), the hops pellets additionally were treated under heat apply in water for an hour ('wort boiling') and the hops' remainder was extracted afterwards (**chapter II, 3**). Simultaneously, the hops sample was also stirred in cold water for the same time. After pYES, an obviously higher amount of the EAC in the hops' remainder extract after heat treatment was observable in comparison to a hops' remainder extract without heat apply, demonstrating the heat-induced formation of the detected EAC, later identified and confirmed as prenylnaringenin (**chapter II, 3**). Additionally, the hops' remainders of four different hop varieties (two varieties from Germany, one variety from Great Britain and one from the United States of America) after the simulation of the wort boiling were investigated by pYES. In all four sample extracts only one EAC was detectable, however, the amount of the EAC differed in the distinct hop varieties (**chapter II, 3**). By comparison of the different applied extracts, the advantages of the RGP-pYES became obvious. On the one hand, the planar chromatographic method is perfectly suitable to see all extracted compounds of a sample, including differences in amounts of these compounds, directly on the plate. On the other hand, the application of the RGP-pYES enables a clear differentiation between zones of native fluorescing compounds of the extracts (blue, red) and zones showing estrogenic activity indicated by the orange fluorescence of resorufin (**chapter II, 3**). An HPTLC method also allows the identification of unknown substances by performing MS detection, as used for the EAC of the hops' remainder extract that thus could be identified and confirmed as prenylnaringenin (**chapter II, 3**).

Furthermore, using the example of the hop flavanone prenylnaringenin, the assumption of the possible determination of EEQ of sample extracts (**chapter II, 2**) was verified, and the term estradiol equivalent amount (EEA) for solid samples was introduced (**chapter II, 3**). For this purpose, different volumes of a hops' remainder extract after heat treatment were applied onto an HPTLC plate and the obtained peak heights of the pYES-signals were plotted against the logarithmized application volumes resulting in sigmoidal curves (**chapter II, 3**). After generating the respective logit-log plot, the EV_{50}-value was determined from the intersection of the linear graph with the abscissa. Thus, the EEQ of the hops'

remainder extract after heat treatment was determined to 39 µg/L and the EEA of the hops' remainder after heat treatment to 52 µg/kg, corresponding to an estrogenic activity of 52 µg 17β-estradiol per kg hops (**chapter II, 3**).

In conclusion, the RGP-pYES represents a fast screening method, which provides the separation of sample components after a simple extraction, including the detection and determination of EAC, while all compounds of the extracts are visible on the plate at one look. At this, the clear differentiation of native fluorescences of sample components and fluorescences of positive signals for estrogenic activity is given (**chapter II, 1 and 3**). The separation of the analytes allows the determination of individual substances, thus, not leading to a sum parameter as result. Moreover, the orange fluorescing zones after pYES indicate the substances which are showing an activity at the human estrogen receptor. The RGP-pYES was successfully applied to water samples and thus demonstrated its suitability as monitoring tool for waters and sewage (**chapter II, 1**). With regard to the watch list of the European Commission, the screening also shows the connection to currently relevant issues. Additionally, also the applicability for other samples such as hops pellets as well as the coupling to MS for the identification of unknowns was demonstrated (**chapter II, 3**). By generation of dose-response curves, moreover, the possibility for an alternative evaluation by means of logit-log plots, the determination of estradiol equivalent factors as well as the estimation of the estrogenic potential of samples or single sample components in terms of estradiol equivalent concentrations and estradiol equivalent amounts was pointed out (**chapter II, 2 and 3**).

The pYES ought to be qualified to investigate further matrices, which has to be verified in future experiments. In addition, by an appropriate selection of analytes and chromatographic conditions, the possibility to execute a targeted analysis of specific substances of interest is given. As example, the analysis of nonylphenols is to be mentioned. The placing on the market, the manufacture and the usages of nonylphenols and nonylphenol ethoxylates is regulated by the European Union as group, why their determination is of great interest. A targeted analysis could take place, for example, by focusing nonylphenol derivatives in a target zone separated from other sample components on the plate, followed by the determination of the total nonylphenols – corresponding to existing regulations – by pYES.

IV Summary

Substances that disrupt or impair the hormone system (endocrine system) or that show an irreversible influence on it are referred to as endocrine disruptors or xenohormones. Concerning this, also estrogen active compounds (EAC) are endocrine disruptors, that are under suspicion of being involved in the formation of tumors or to induce disruption during development and reproduction, and are, for example, blamed for being responsible for the feminization of fish. At this, EAC can be natural (human, phytoestrogens) but also synthetic substances, which are discharged to the environment by humans (e.g. pharmaceuticals, pesticides, additives). Regarding the ubiquitous presence of EAC and with respect to the watch list of substances of the European Commission for Union-wide monitoring in the field of water policy of the year 2015 (includes four EAC, *inter alia* ethinylestradiol EE2, estradiol E2 with a maximum acceptable method detection limit of 0.4 ng/L), suitable methods for the analysis of EAC are required. For that purpose, several *in vivo* and *in vitro* methods are available by which, however, often only the effect on the organism and the whole bioactivity of a sample, respectively, are considered, and in their course no determination or identification of single EAC takes place. The YES assay (yeast estrogen screen), for example, is a cell-based method that is executed in liquid solutions in microtiter plates and works with genetically modified yeasts, which contain the human estrogen receptor (hER) and a reporter gene encoding for the enzyme β-galactosidase. In presence of EAC, the enzyme is produced and subsequently cleaves a substrate that is used to measure the receptor activity and thus the estrogenic activity. The transfer of the YES assay to high-performance thin-layer chromatography (HPTLC) was successfully demonstrated and advanced, thus resulting in the combination of a chromatographic separation of analytes and the detection of EAC using genetically modified yeast cells directly on the HPTLC plate (HPTLC planar yeast estrogen screen, HPTLC-pYES). Usually, the substrate 4-methylumbelliferyl-β-D-galactopyranoside is used, releasing blue fluorescing 4-methylumbelliferone (MU) after enzymatic cleavage as positive signal for estrogenic activity. Samples of interest to be investigated, however, often contain plenty of components, partly showing native fluorescences (blue, red), why the detection of the blue fluorescing MU can be interfered.

The objective of the presented research work was to introduce a suitable substrate, which allows the detection of EAC without interferences by a clear differentiation between positive signals due to estrogenicity and signals due to native fluorescences. Applying this substrate, a pYES should be developed, allowing a fast screening for EAC in water and sewage samples, while single components are separated from each other by HPTLC and a possible

estrogenic activity of the single substances can be detected directly on the HPTLC plate. The single EAC should be identified with suitable standard substances or by coupling to mass spectrometry (MS).

By applying the substrate resorufin-β-D-galactopyranoside (RGP) and by using automated devices, the RGP-pYES as fast screening tool for EAC was developed in the course of this research work and was successfully applied to waste water samples and extracts of hops pellet samples. A screening method using HPTLC simultaneously represents a planar clean-up, why samples do not have to undergo complex steps of sample preparation or purification. A chromatographic separation in combination with the detection of estrogenic activity using genetically modified yeasts directly on the plate allowed the detection, the determination and the identification of single EAC. Using RGP, which releases orange fluorescing resorufin after enzymatic cleavage, enabled to clearly differentiate between fluorescences due to estrogenicity and the native fluorescence of sample components. Application of the RGP-pYES to spiked water samples and sewage samples showed high recovery rates (close to 100% for both E2 and EE2 with relative standard deviations ≤25%) and a good precision (E2 in the waste water influent with a concentration of ~17 ng/L, relative standard deviation of 11%, n = 4), and thus the applicability of the screening as monitoring tool for environmental samples and the connection to current issues (watch list; detection limit for E2 was determined to 0.4 ng/L).

By means of the developed pYES and suitable evaluation methods, additionally the option to generate dose-response curves of known and unknown EAC was given and the generation of so-called logit-log plots was possible. This enabled the determination of estradiol equivalent factors of known EAC and also the determination of estradiol equivalent concentrations and amounts, respectively, of known and unknown EAC in liquid and solid samples. Thus, the possibility to estimate the estrogenic potential of a sample or single sample components was given. The coupling of pYES to MS additionally allowed the identification of unknown EAC, demonstrated exemplarily by investigation of extracts of hops pellet samples, in which the only detected EAC in the hops extracts was identified as prenylnaringenin.

Since the pYES is a method that uses a planar system, it provides the advantage to see all chromatographically separated sample components at one look. Additionally, pYES provides the evidence of a possible estrogenic activity (activity at the hER) of single substances, when a differentiation between native occurring fluorescences of sample contaminants and the fluorescence as positive signal for estrogenicity of a substance is granted.

V Zusammenfassung

Stoffe, welche das Hormonsystem (endokrine System) irreversibel beeinflussen, stören oder beeinträchtigen, werden als endokrine Disruptoren oder auch als Xenohormone bezeichnet. Hierzu zählen auch östrogen wirksame Stoffe (estrogen active compounds, EAC), welche unter anderem im Verdacht stehen bei der Tumorbildung beteiligt zu sein oder Störungen in der Entwicklung und Reproduktion hervorzurufen, und beispielsweise für die Verweiblichung von Fischen verantwortlich zu sein. EAC können hierbei natürlichen Ursprungs (menschlich, pflanzlich) sein oder synthetisch hergestellte Stoffe, welche durch den Menschen in die Umwelt eingetragen werden (z.B. Arzneimittel, Pestizide, Additive). Aufgrund des ubiquitären Vorkommens von EAC und der 2015 von der Europäischen Kommission veröffentlichten Beobachtungsliste (watch list) für die unionsweite Überwachung im Rahmen der Wasserpolitik (beinhaltet vier EAC, u.a. Ethinylestradiol EE2, Estradiol E2 mit einer höchstzulässigen Nachweisgrenze NG von 0,4 ng/L) sind geeignete Methoden zur Untersuchung auf EAC erforderlich. Hierfür stehen einige *in vivo* und *in vitro* Verfahren zur Verfügung, wobei jedoch häufig nur die Wirkung auf den Organismus bzw. die gesamte Bioaktivität der Probe betrachtet wird und keine Bestimmung oder Identifizierung der einzelnen EAC erfolgt. Ein Beispiel einer zellbasierten Methode ist der YES-Assay (yeast estrogen screen), welcher in flüssigen Medien in Mikrotiterplatten durchgeführt wird und mit genetisch modifizierten Hefen arbeitet, die den humanen Östrogenrezeptor (human estrogen receptor, hER) und ein Reportergen enthalten, welches für das Enzym β-Galactosidase kodiert. In Anwesenheit von EAC wird das Enzym produziert und setzt anschließend ein Substrat um, welches zur Messung der Rezeptor- und damit der Östrogenaktivität dient. Die Übertragung des YES-Assay auf die Hochleistungs-Dünnschichtchromatographie (high-performance thin-layer chromatography, HPTLC) wurde erfolgreich gezeigt und weiterentwickelt, so dass eine chromatographische Trennung der Analyten mit dem Nachweis der EAC unter Verwendung genmodifizierter Hefezellen direkt auf der HPTLC-Platte kombiniert werden konnte (HPTLC planar yeast estrogen screen, HPTLC-pYES). Hierbei findet für gewöhnlich das Substrat 4-Methylumbelliferyl-β-D-Galactopyranosid seinen Einsatz, welches nach enzymatischer Spaltung blau fluoreszierendes 4-Methylumbelliferon (MU) als positiven Nachweis östrogener Aktivität freisetzt. Häufig beinhalten die zu untersuchenden Proben jedoch viele Begleitkomponenten, welche zum Teil native Fluoreszenzen aufzeigen (blau, rot), wodurch der Nachweis des blau fluoreszierenden MU gestört werden kann.

Ziel dieser Forschungsarbeit war es, ein geeignetes Substrat zu finden, welches die störungsfreie Detektion von EAC ermöglicht, indem Positivsignale deutlich von nativen Fluoreszenzen unterschieden werden können. Mittels dieses geeigneten Substrates sollte

ein pYES entwickelt werden, welcher ein schnelles Screening auf EAC in Wasser- bzw. Abwasserproben ermöglicht, wobei Einzelkomponenten mittels HPTLC getrennt werden und eine mögliche östrogene Wirksamkeit einzelner Substanzen direkt auf der HPTLC-Platte nachgewiesen wird. Einzelne EAC sollten hierbei durch geeignete Standardsubstanzen oder durch Kopplung mit Massenspektrometrie (MS) identifiziert werden können.

Durch die Wahl des Substrates Resorufin-β-D-Galactopyranosid (RGP) und unter Verwendung automatisierter Geräte konnte im Verlauf dieser Forschungsarbeit der RGP-pYES als schnelles Screening auf EAC entwickelt und erfolgreich auf Abwasserproben und Extrakte von Hopfenpellets angewandt werden. Ein Screening unter Einsatz der HPTLC stellt gleichzeitig ein planares Clean-up dar, wodurch die Proben keinen aufwändigen Reinigungs- oder Aufarbeitungsschritten unterzogen werden müssen. Die chromatographische Trennung in Kombination mit dem Nachweis östrogener Aktivität mittels genmodifizierter Hefen direkt auf den Platten ermöglichte den Nachweis, die Bestimmung und die Identifizierung einzelner EAC. Die Verwendung des RGP, welches durch enzymatische Spaltung orange fluoreszierendes Resorufin freisetzt, erlaubte hierbei eine klare Differenzierung von Fluoreszenzen aufgrund östrogener Wirksamkeit gegenüber nativer Fluoreszenz von Probenkomponenten. Die Anwendung des RGP-pYES auf Extrakte von gespikten Wasserproben und Abwasserproben zeigte hohe Wiederfindungsraten (~100% für E2 und EE2, relative Standardabweichungen RSD ≤25%) und eine gute Präzision (E2 ~17 ng/L im Abwasserzufluss, RSD 11%, n = 4) und damit die Eignung des Tests als Monitoring-Tool für Umweltproben, und den Bezug zu aktuellen Fragestellungen (watch list; NG für E2 zu 0,4 ng/L bestimmt). Mittels des entwickelten pYES und geeigneter Auswerteverfahren war es zudem möglich, Dosis-Wirkungs-Kurven bekannter und unbekannter EAC und damit sogenannte logit-log Plots zu erstellen. Dies ermöglichte die Bestimmung von Estradiol-Äquivalenz-Faktoren bekannter EAC sowie die Ermittlung von Estradiol-Äquivalenz-Konzentrationen bzw. -Gehalten bekannter und unbekannter EAC in flüssigen und festen Proben, womit die Möglichkeit der Abschätzung des östrogenen Potentials einer Probe oder einer einzelnen Probenkomponente gegeben ist. Die Kopplung des pYES mit der MS erlaubte zusätzlich die Identifizierung unbekannter EAC, wie am Beispiel der Untersuchung von Extrakten von Hopfenpellets gezeigt werden konnte, in welchen Prenylnaringenin als einzig nachweisbare EAC der Hopfenextrakte identifiziert wurde. Als Methode im planaren System bietet der pYES den Vorteil, alle chromatographisch getrennten Probenbestandteile auf einen Blick zu sehen, und zusätzlich durch den Biotest eine mögliche östrogene Aktivität (Aktivität am hER) der Einzelsubstanzen anzuzeigen. Hierbei kann zwischen nativ auftretenden Fluoreszenzen der Probenkomponenten und der Fluoreszenz als Positivsignal der östrogenen Wirksamkeit einer Substanz differenziert werden.