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Yeast Estrogen Screen Assay as a Tool for Detecting Estrogenic Activity of Waters

Mirjana Bistan[1], Mojca Podgorelec[1#], Romana Marinšek Logar[2] and Tatjana Tišler[1]*

[1]Laboratory for Environmental Sciences and Engineering, National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia
[#]Present address: LEK d.d., Trimlini 2d, SI-9220 Lendava, Slovenia
[2]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia

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Summary

The presence of endocrine disrupting compounds in wastewaters, surface waters, groundwater and even drinking waters has become a major concern worldwide, since they negatively affect wildlife and humans. Therefore, these substances should be effectively removed from effluents before they are discharged into surface waters to prevent pollution of groundwater, which could be a source of drinking water. Furthermore, an efficient control of endocrine disrupting compounds in wastewaters based on biological and analytical techniques is required. In this study, a "yeast estrogen screen" (YES) bioassay was introduced and optimized with the aim to assess potential estrogenic activity of waters. At first, assay duration, volume of added substrate to the test medium and wavelength used to measure the substrates' absorbance were estimated. Several compounds, such as 17β-estradiol, 17α-ethinylestradiol, bisphenol A, nonylphenol, genisteine, hydrocortisone, dieldrin, atrazine, methoxychlor, testosterone and progesterone were used to verify its specificity and sensitivity. The optimized YES assay was sensitive and responded specifically to the selected estrogenic and non-estrogenic compounds in aqueous samples. Potential estrogenicity of influent and effluent samples of two wastewater treatment plants was assessed after the samples were concentrated by solid-phase extraction (SPE) procedure using Oasis® HLB cartridges and methanol as eluting solvent. Up to 90 % of relative estrogenic activity was

[*]Corresponding author; Phone: ++386 1 476 2287; Fax: ++386 1 476 3000; E-mail: tatjana.tisler@ki.si

detected in concentrated samples of influents to wastewater treatment plants and estrogenic activity was still present in the concentrated effluent samples. We found that the introduced YES assay is a suitable screening tool for monitoring the potential estrogenicity of effluents, which are discharged into surface waters.

Key words: endocrine disrupting compounds, estrogenic activity, monitoring, solid-phase extraction, yeast estrogen screen assay, Waters

Introduction

Endocrine disrupting compounds (EDCs) comprise a wide range of natural and synthetic compounds, which exhibit a potential to elicit negative effects on endocrine systems of humans and wildlife. The group of EDCs includes natural and synthetic estrogen hormones, pharmaceuticals, pesticides, industrial chemicals, such as PAHs, PCB, surfactants and plasticizers, and heavy metals (*1-4*). Natural and synthetic estrogens generally display much stronger estrogenic effects than phyto- and xenoestrogens. However, the concentrations of the latter compounds in the aquatic environment are usually several orders of magnitude higher than those of estrogens (*4,5*).

Recent studies have reported the presence of EDCs in wastewaters, surface waters, groundwater and even drinking waters worldwide at the concentrations higher than those, which are already estrogenically active and for this reason these findings are a cause for concern (*6,7*). Human population is exposed to EDCs in food, water and environment. Recent reviews and scientific consensus statements find the evidence for adverse reproductive outcomes i.e. infertility, cancers, malformations, and effects on other endocrine systems from exposure to EDCs (*5,8,9*). Therefore, efficient wastewater treatment technologies and control of effluents quality are very important steps in prevention pollution of drinking water by EDCs. Traditionally, quality control of effluents is mainly based on analytical measurements of target compounds such as EDCs. However, environmental samples are almost always complex mixtures of known and unknown chemicals, which make complete identification and quantification of all compounds present, as well as by-products or metabolites, unfeasible and economically unacceptable. Furthermore, adverse biological effects caused by EDCs can not be measured by chemical analyses. For this reason, a biological assay, such as *"in vitro"* yeast estrogen screen assay (YES assay), is required for screening the estrogenic activity of environmental samples, *i.e.* effluents, wastewaters and landfill leachates (*3,4,7,10,11*). An

estrogenic activity identification and evaluation procedure by means of YES assay was described in many papers (*5,12-14*).

Since the amounts of some EDCs in the environment are at trace levels, samples must undergo extraction and concentration procedures to reach the levels of detection (*15*). Several solid-phase extraction (SPE) procedures for extraction and concentration of wastewaters have been described by authors, such as Ballesteros *et al.* (*16*), Janex-Habibi *et al.* (*17*), Yang *et al.* (*18*), etc.

The aim of this study was to optimize the *"in vitro"* YES assay as a tool for detecting estrogenic activity of aqueous samples. For this purpose, some important parameters were investigated, such as time of exposure, absorbance maximum and concentration of the chromogenic substrate β-D-galactopyranoside (CPRG) added to test medium. Specificity of the assay was assessed by several known estrogenic compounds. Considering detecting estrogenic activity of water samples, a SPE procedure was optimized for extraction and concentration of estrogenic active compounds from aqueous samples. Finally, the introduced YES bioassay was used to investigate estrogenic activity of influents and effluents from two municipal wastewater treatment plants (WWTPs) in Slovenia.

Methods

Sample preparation

Compounds 17β-estradiol (E2), 17α-ethinylestradiol (EE2), bisphenol A (BPA), nonylphenol (NP), genisteine (G), hydrocortisone (HC), dieldrin (D), atrazine (A), methoxychlor (M), testosterone (T) and progesterone (P) were purchased from Sigma-Aldrich (Germany). Samples for testing specificity and sensitivity of YES assay were prepared in 1 mL of 99 % ethanol (Fluka, Germany). Working concentrations of compounds were 27.2 µg/L for E2, 29.6 µg/L for EE2, 0.2 mg/L for BPA and G, 93.7 µg/L for NP, 36.2 µg/L for HC, 10 g/L for D, A and M, 28.8 µg/L for T and 31.4 µg/L for P. Concentrations of water samples of E2, EE2 and BPA used in solid phase extraction (SPE) procedure were equal to 272 µg/L, 27.2 µg/L and 2.72 µg/L for E2, 296 µg/L, 29.6 µg/L and 2.96 µg/L for EE2 and 0.2 g/L, 0.1 g/L and 0.05 g/L for BPA and 0.2 g/L for G. Stock concentration of substrate β-D-galactopyranoside (CPRG) was 10 mg/mL.

YES assay procedure

A recombinant yeast strain *Saccharomyces cerevisiae* BJ1991 used in this experiment was kindly provided to us from Professor John P. Sumpter (Genetics Department of Glaxo Corporation, UK). Yeast hosts an integrated gene coding for human estrogen receptor (hER) in its genome and expression plasmids carrying the reporter gene *lac-Z* (encoding the enzyme β-galactosidase). Following the activation of *lac-Z* gene in the presence of estrogenic active compounds, β-galactosidase degrades substrate CPRG (*13*).

Preparation of medium components

All the ingredients were purchased from Sigma-Aldrich (Germany). Minimal medium and growth medium were prepared following Routledge and Sumpter (*13*). The growth medium was inoculated with 0.25 mL of the concentrated stock yeast and incubated at 28 °C for approximately 24 h on an orbital shaker (150 rpm) until A_{620}=1.0 was reached. The assay medium was prepared by adding 200 μL of the chromogenic substrate CPRG to 50 mL fresh growth medium and seeded with 2 mL yeast from a 24-h yeast culture. Serial dilutions of compounds were prepared in ethanol and 10 μL aliquots of these solutions were transferred to 96-well flat-bottom microtiter plates in sterile conditions. After ethanol dried, the yeast cells in assay medium were added to each hole on the microtiter plate. Then, microtiter plates were incubated at 34 °C for 48-52 hours. The absorbance of each sample was measured on the microtiter plate reader PowerWave XS (BioTek, USA). On each microtiter plate a positive, negative and blank controls were used. Since 17β-estradiol (E2) is the main natural human estrogen, it was used as a positive control. On the other hand, testosterone (T) and progesterone (P) are natural human androgen hormones without the ability of binding to the human estrogen receptor; therefore, they were used as a negative control. As a blank control (B), yeasts exposed to the growth medium with substrate CPRG were used in order to detect whether yeasts themselves, without exposing to estrogen active compound, could degrade CPRG.

YES assay optimization

The YES assay was optimized for incubation time (by the absorbance measurements at different time intervals), concentration of the substrate CPRG in the test medium (10, 20, 40, 60 and 80 μg/mL) and the maximal absorption wavelength for degraded CPRG substrate (scan in a range from 500 to 600 nm), by using compounds E2, EE2 and BPA.

Estrogenic activity

For calculating estrogenic activity (EA), the absorbance (*A*) measurements at 575 and 620 nm were carried out and EA was expressed as the activity of enzyme β-galactosidase (eq. /1/). $A(575\ nm)_{sample}$ represents the absorbance of sample measured at 575 nm, $A(620\ nm)_{sample}$ represents the absorbance of sample measured at 620 nm, while $A(620\ nm)_{blank}$ represents the turbidity of yeast in the assay medium. Equation adopted by Fent *et al.* (*12*) reads:

$$\beta - galactosidase\,activity\ (EA) = A(575nm)_{sample} - \left[A(620nm)_{sample} - A(620nm)_{blank}\right] \qquad /1/$$

Solid- phase extraction procedure (SPE procedure)

Optimization of the SPE procedure involved testing of two different SPE cartridges Supelclean™ ENVI 18-SPE (Supelco, USA) and Oasis® HLB SPE (Milford, USA), and eluting solvents methanol and ethyl acetate. Conditioning of cartridges was performed with 4 mL of methanol or ethyl acetate, followed by 4 mL of distilled water. After loading the samples, the cartridges were washed with 5 % methanol or 5 % ethyl acetate. During these steps vacuum was maintained. Then cartridges were dried under a gentle stream of nitrogen (N_2) and compounds were eluted with 4 mL of methanol or ethyl acetate and collected in test tubes. Eluted samples in test tubes were exposed to the gentle stream of N_2 for concentrating the samples to the volume of 1 mL and stored at -20 °C before further used. Recovery of compounds E2, EE2, BPA and G was calculated using equation /2/. Samples` estrogenic activity ($EA_{sample}$) and samples` estrogenic activity after SPE procedure ($EA_{SPEsample}$) were calculated using equation /1/.

$$recovery\ (\%) = \frac{EA_{SPE\ sample}}{EA_{sample}} \times 100 \qquad /2/$$

Wastewater samples

Influent and effluent grab samples from two municipal wastewater treatment plants (WWTPs) were collected in 5 l plastic bottles. Collected wastewater samples were processed immediately upon arrival. Pretreatment of samples (250 mL) involved centrifugation at 5000 rpm for 10 min and filtration through the 1.2 and 0.3 μm membrane filters (*16*). This was followed by the SPE procedure, where methanol and ethyl acetate were used as conditioning

and eluting solvents on the same SPE cartridge (ethyl acetate was following methanol by conditioning and eluting). Extracted and concentrated samples were stored at -20 °C before used in YES assay.

The estrogenic activity of wastewater samples was expressed as the activity of enzyme β- galactosidase calculated by using equation /1/ and the relative estrogenic activity (REA, %) was calculated using equation /3/, adopted by Cajthaml (*19*), which reads:

$$REA\ (\%) = \frac{EA_{\ sample} - EA_{\overline{blank}}}{\Delta} \times 100\ , \qquad /3/$$

where EA_{sample} is samples` estrogenic activity calculated using eq. /1/, EA_{blank} is average of blank control estrogenic activity calculated using eq. /1/ and Δ is an interval of estrogenicity with the initial point in the highest EA_{E2} value and final point in EA_{blank}.

Enzymatic deconjugation

For activation of conjugated estrogens, deconjugation step has to be performed. According to Mouatassim-Souali (*20*) the wastewaters extract from SPE procedure was evaporated to dryness under N_2-gas. Then, the samples were re-dissolved in 1 ml 0.1 M acetic acid buffer (pH 5) containing β- glucuronidase enzyme with minimum 2000 units glucuronidase activity and 150 units sulphatase activity (HP-2 from *Helix pomatia* (Sigma, Germany)). After 24 hours incubation at 40 °C the reaction of deconjugation was terminated by addition of acidified water (pH 3). The incubation mixture was then extracted on preconditioned Oasis HLB® SPE cartridge as described previously. Estrogenic activity and relative estrogenic activity were calculated using eq. /1/ and /3/.

Results

YES assay procedure optimization

Optical density of yeast culture suitable to use in the assay was at OD_{620}=1. This was reached between 18 and 24 hours of incubation at 28 °C on an orbital shaker at 140 rpm. Furthermore, the YES assay optimization showed that yeast should be exposed to tested samples between 48 and 58 hours in order to obtain data with the best contrast (Fig. 1). When the YES assay is prolonged the CPRG degradation product might act as estrogenic active compound and therefore false positive results could be obtained, which was also confirmed by Vanderperren *et al. (21)*.

Fig. 1.

Scan of visible light absorption in a wavelength range from 500 to 600 nm showed the highest obtained absorbance at wavelength 575 nm (data not shown); and for the best results obtained at economically spent substrate, 200 µl of CPRG should be added to the assay medium (Fig. 2).

Fig. 2.

Specificity and sensitivity of YES assay

Specificity and sensitivity of YES assay were studied using compounds E2, EE2, BPA, NP, G, HC, D, A, M, T and P (see Figure 3 and Table 1). The obtained results show that E2, EE2, BPA, NP, G and M possess estrogenic activity, whereas other tested compounds do not bind to the estrogen receptor (Figure 3). Sensitivity of tested compounds is presented according to Kuruto-Niwa *et al.* (*22*) as concentrations, at which the lowest (a), 50 % of the highest (b) and the highest (c) estrogenic effect is observed (Table 1).

Fig. 3.

Table 1.

SPE procedure optimization

In general, the obtained recoveries for both cartridges were in the same range for all tested compounds (Table 2). The obtained recoveries using methanol and ethyl acetate on the Supelclean™ ENVI 18-SPE cartridge were similar. However, we observed some white turbidity of the ethyl acetate when we dropped it into holes of the microtiter plate in the YES assay. It seems that ethyl acetate reacts with plastic of the microtiter plate. For this reason, further experiments of the SPE procedure optimisation were conducted only with methanol. In the SPE procedure of wastewaters samples ethyl acetate in the vials was dried out under gentle stream of nitrogen and then replaced by methanol.

Some additional concentrations of E2, EE2 and BPA were tested by using Oasis® HLB cartridges (Figure 4).

Table 2.

Fig. 4.

Wastewater samples

Influent and effluent samples examined by YES assay indicated estrogenic activity as described in Tables 3 and 4. When yeast growth was inhibited for 50 % or more due to toxic compounds present in tested samples, the REA was not calculated due to possible false results.

Influent concentrates of wastewater A eluted by methanol (A-influent-MeOH) caused high inhibition (up to 90 %) of yeast growth and consequently REA was not measured (n.d.). When influent concentrates eluted by ethyl acetate (A-influent-EtAc) up to 62 % of REA was determined before deconjugation, but after deconjugation step the REA increased up to 93 %. Although effluent concentrates were less toxic than influents, similar REA was determined. However, REA stayed at the same level or even slightly decreased in effluent concentrates after deconjugation; this could be explained by deconjugation of estrogens during treatment processes in WWTP and/or looses of EDCs due to repeating SPE procedures.

Table 3.

Wastewater B influent and effluent concentrates revealed lower toxicity manifested in growth inhibition of yeast and REA in comparison to wastewater A influent and effluent samples (Table 4). No estrogenic activity was detected neither in influent concentrates (the concentrates with growth inhibition lower than 50 %) nor in effluent concentrates samples. However, after deconjugation step up to 46 % and 48 % of REA was determined in influent and effluent concentrates, respectively.

Table 4.

Discussion

In the present work, the YES assay and SPE procedure were optimized for testing estrogenic activity of water samples. Firstly, the specificity of YES assay was tested for the selected chemicals. Specificity of YES assay is explained by the nature of the estrogen receptor, where ligand-binding site is larger and more flexible than the 17β-estradiol molecule requires (*23*,*24*). This makes the estrogen receptor more vulnerable as a target for interference by many different compounds with analogy in chemical structure, such as synthetic estrogen hormone (EE2), industrial chemical (BPA, NP), phytoestrogen (genisteine) or pesticide (methoxychlor). On the other hand, homology does not play any role, since from group of steroid hormones 17β-estradiol (E2), hydrocortisone (HC), testosterone (T) and progesterone (P), by the common origin from cholesterol, only E2 caused estrogenic activity (with the ability of binding with the estrogen receptor). All chemicals showed an activation of the estrogen receptor in a concentration-dependent manner. The observed sensitivity of YES assay for natural and synthetic estrogens is in the same range (15 ng/L), whereas sensitivity of YES assay for nonylphenol (NP) is 5-times lower (73 ng/L), for bisphenol A (BPA) and genisteine (G) is more than 5.000 times lower (78 μg/L for BPA and 312 μg/L for G) and for methoxychlor (M) is more than 100.000 times lower (2 mg/L). The presented results are comparable to previously described results from Routlegde and Sumpter (*13*), Daston *et al.* (*25*), Giesy *et al.* (*26*) and Ternes *et al.* (*27*).

In our case, the incubation time of YES assay was between 48 and 58 hours, the absorbance of degraded CPRG substrate was measured at wavelength 575 nm as it provides about 75 % higher response in comparison to the measurements at 540 nm, and with the volume of added substrate CPRG to the assay medium equal to 200 μl. While Routledge and Sumpter (*13*) represented estrogenic activity as the absorbance of degraded substrate CPR measured at 540 nm, in our work estrogenic activity was presented as the activity of β-galactosidase calculated by equation [1].

Introducing and optimizing the SPE procedure, the sorbent cartridge Oasis® HLB and the use of methanol as eluting solvent, were found to be the most optimal. Recovery of SPE procedure is comparable to previously reported data from Ballesteros *et al.* (*16*) and Lopez de Alda and Barcelo (*28*) and was above 60 % in all cases. The highest recovery (above 90 %) was obtained at the highest tested concentrations followed by the medium concentration (recovery above 80 %). As expected, the lowest recovery (above 60 %) was found at the lowest tested concentrations of the examined compounds E2, EE2 and BPA.

Concentrated samples of influent and effluent examined for estrogenic activity indicated up to 90 % of relative estrogenic activity (REA). In general, EA of influents was higher than EA determined in effluents, which could be explained by removal of estrogens and xenoestrogens during treatment processes in WWTPs (*29,30,31*). Furthermore, the EA of the tested samples after deconjugation step increased in most cases in comparison to the original samples, which means that present estrogens were in inactive (conjugated) form and became active only after deconjugation. This was not the case for the effluent sample obtained from the WWTP A (A-effluent-EtAc) as lower EA level was obtained when concentrates eluted with ethyl acetate. This could be explained by possible elimination of estrogenic compounds from water samples due to repeated SPE procedures before and after deconjugation step.

Conclusions

The obtained results showed that the optimized YES assay was sensitive and specifically responded to the selected estrogenic and non-estrogenic compounds in water samples. The introduced YES assay was then applied to assess the estrogenicity of concentrated influent and effluent samples from two municipal WWTPs. Up to 90 % of relative estrogenic activity of influent and effluent samples was detected, which was probably due to the presence of natural and synthetic estrogen hormones and xenoestrogens from households. Less estrogenic activity was found in effluent and influent samples of the WWTP B (up to 48 %) in comparison to the samples obtained from the WWTP A (up to 93 %). However, both WWTPs were partially successful in removing EDCs, since estrogenic activity was still present in most concentrated effluent samples.

We can conclude that the introduced YES assay is a suitable screening tool for monitoring the potential estrogenicity of effluents, which are discharged into surface waters. Only effective removal of EDCs from wastewaters using appropriate treatment processes could protect the aquatic environment reliably from pollution with these compounds and consequently prevent adverse effects on wildlife and humans.

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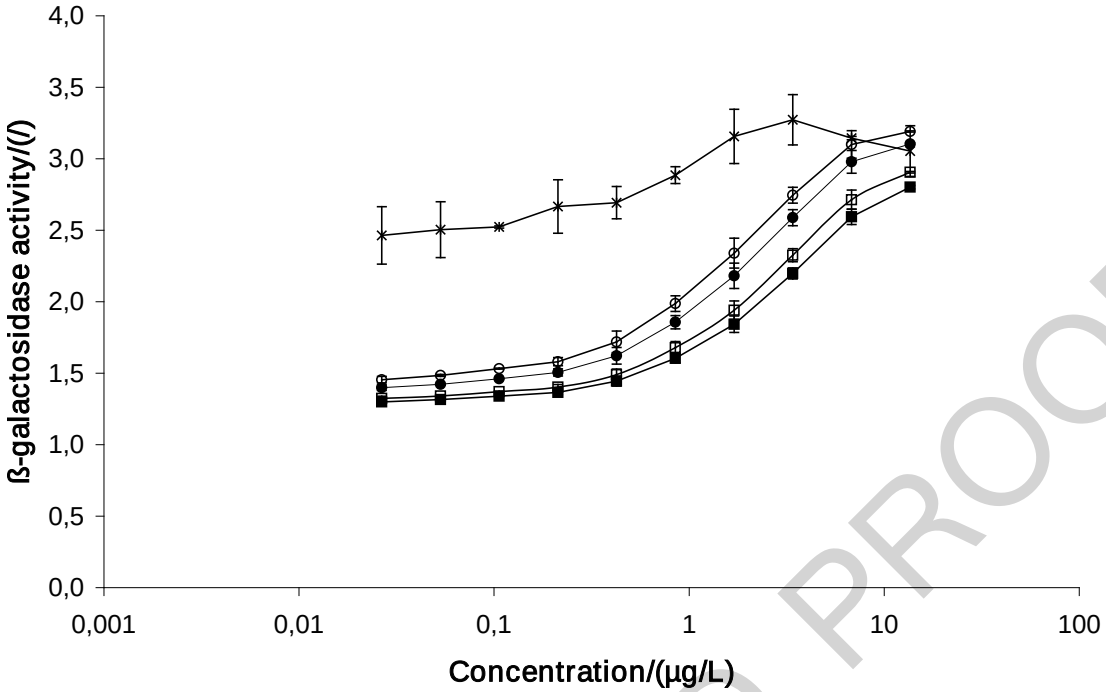

Fig. 1. Optimization of incubation time using standard E2; (—■—) 48 h, (—□—) 51 h, (—●—) 55 h, (—○—) 59 h, (—✶—) 72 h. (Arithmetic means and standard deviation (n = 2) of activity of β-galactosidase are presented)

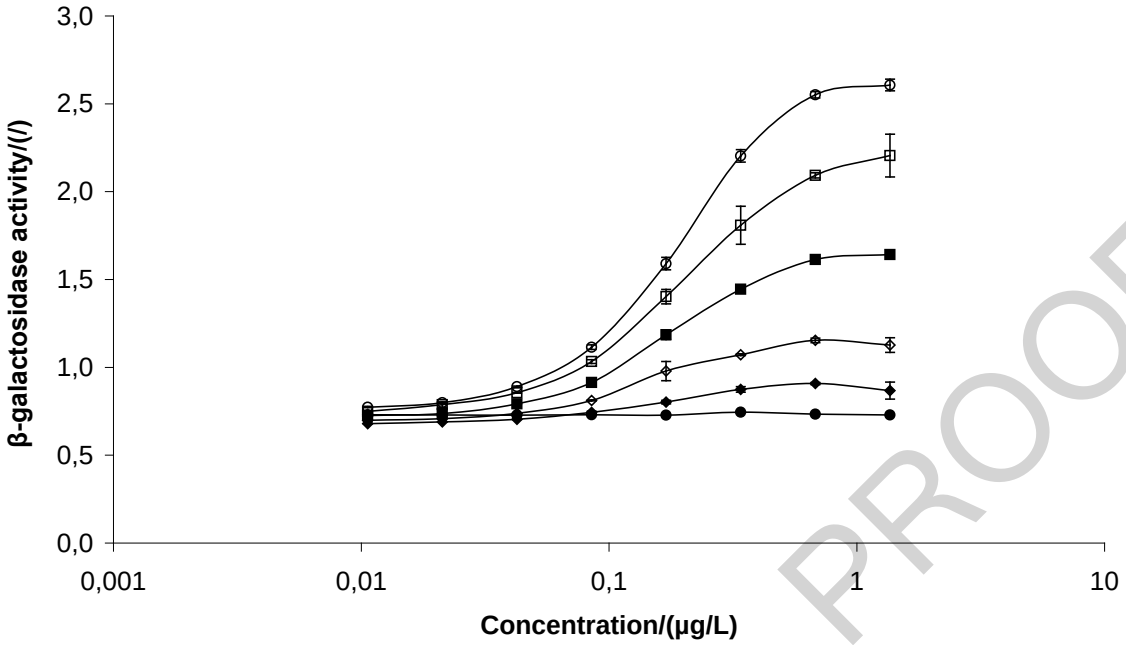

Fig. 2. Optimization of CPRG concentration in the assay medium; (—○—) 80 µg/mL, (—□—) 60 µg/mL, (—■—) 40 µg/mL, (—◇—) 20 µg/mL, (—◆—) 10 µg/mL, (—●—) B. (Arithmetic means and standard deviation (n = 2) of activity of β-galactosidase are presented)

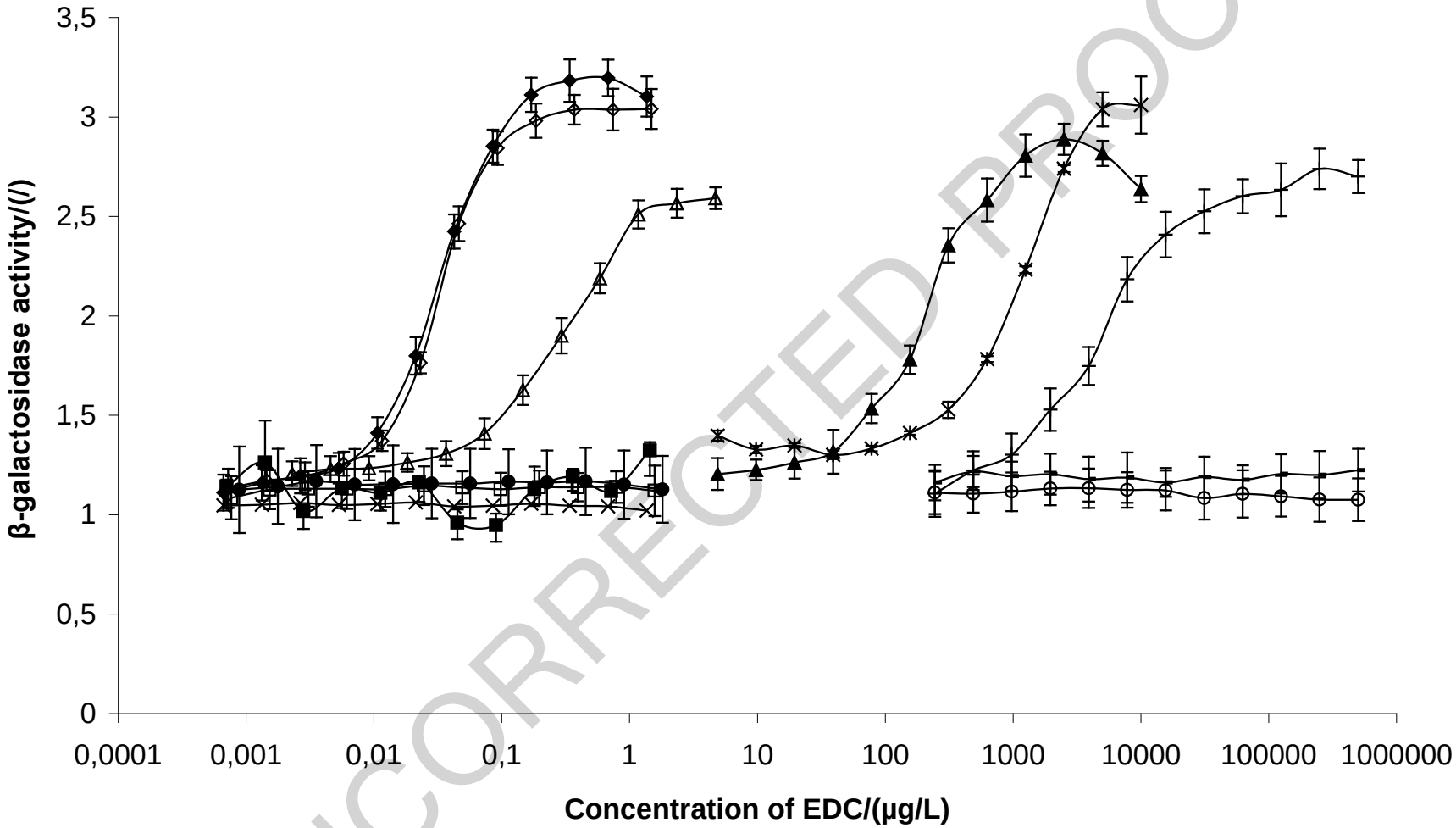

Fig. 3. Specificity of YES assay for (◆) E2, (◇) EE2, (▲) BPA, (△) NP, (✳) G, (●) HC, (+) M, (-) D, (○) A, (■) T, (□) P and (×) B. (Arithmetic means and standard deviation (n = 2) of activity of β-galactosidase are presented)

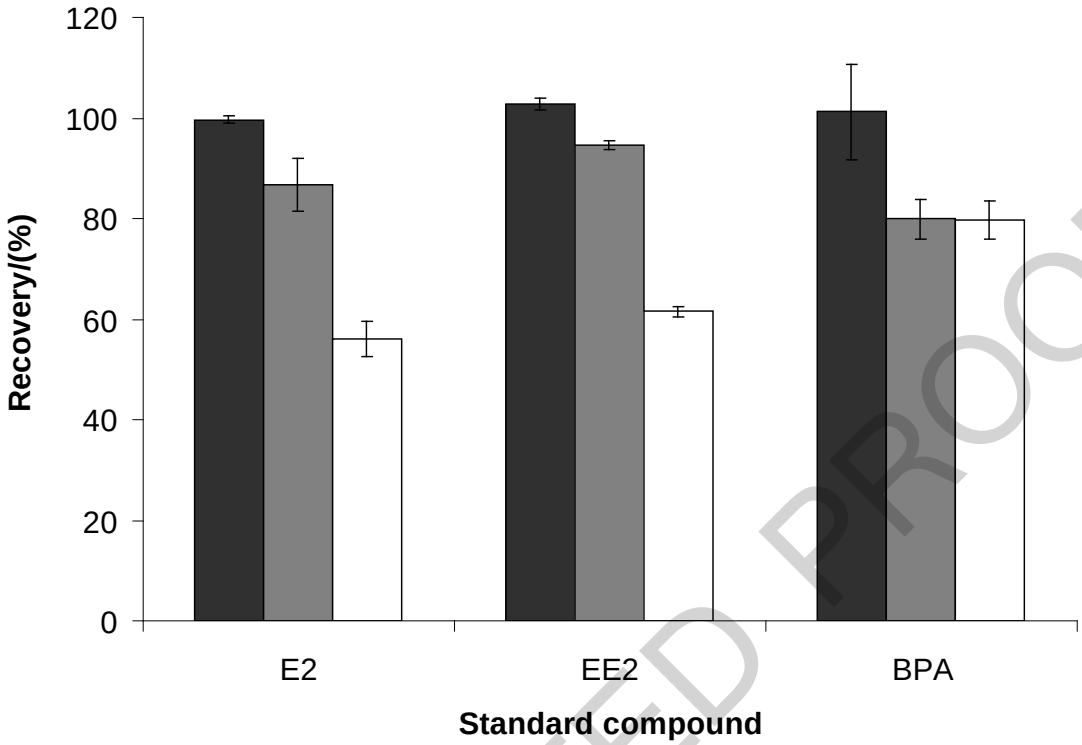

Fig. 4. Recovery of SPE procedure for E2, EE2 and BPA; (■) concentration 13.6 μg/L for E2, 14.8 μg/L for EE2 and 10 mg/L for BPA, (■) concentration 1.36 μg/L for E2, 1.48 μg/L for EE2 and 5 mg/L for BPA and (□) concentration 0.136 μg/L for E2, 0.148 μg/L for EE2 and 2.5 mg/L for BPA (Arithmetic means and standard deviation (n=16) of recovery calculated from activity of β-galactosidase are presented)

Table 1. Sensitivity of YES assay for estrogenic active compounds

Compound	**[a]Min. effect conc. (µg/L)**	**[b]EC_{50} (µg/L)**	**[c]Max. effect conc. (µg/L)**
E2	0.015 ± 0.002	0.044 ± 0.004	0.34 ± 0.03
EE2	0.015 ± 0.002	0.042 ± 0.004	0.37 ± 0.04
Bisphenol A	78 ± 7	337 ± 30	5000 ± 100
Nonylphenol	0.073 ± 0.007	0.32 ± 0.03	1.17 ± 0.1
Genisteine	313 ± 30	1290 ± 90	5000 ± 150
Methoxychlor	1953 ± 200	11400 ± 800	250000 ± 2000

[a]Concentration at which the lowest estrogenic effect was observed

[b]Concentration at which 50 % of the highest estrogenic effect was observed

[c]Concentration at which the highest estrogenic effect was observed

Table 2. Recovery (%) of E2 (13.6 µg/L), EE2 (14.8 µg/L), bisphenol A (10 mg/L) and genisteine (10 mg/L) using different SPE cartridges and eluting solvents (Arithmetic means and standard deviation (n=10-20) are presented)

Compound	Recovery (%)		
	ENVI 18		Oasis® HLB
	Methanol	Ethyl acetate	Methanol
E2	99.3 ± 0.7	95.2 ± 4.8	99.3 ± 0.7
EE2	100.8 ± 1.0	83.3 ± 16.7	91.0 ± 9.0
Bisphenol A	97.4 ± 2.6	95.5 ± 4.5	91.4 ± 8.6
Genisteine	100.6 ± 0.6	90.4 ± 9.6	83.0 ± 17

Table 3. Relative estrogenic activity (REA, %) of A influent and A effluent

	Before deconjugation			
concentrate (-x)	A-influent-MeOH	A-influent-EtAc	A-effluent-MeOH	A-effluent-EtAc
12.5	n.d.	62.1	n.d.	57.1
6.3	n.d.	59.2	37.7	51.6
3.1	n.d.	47.8	32.8	34.7
1.6	n.d.	19.6	14.6	0
	After deconjugation			
concentrate (-x)	A-influent-MeOH	A-influent-EtAc	A-effluent-MeOH	A-effluent-EtAc
12.5	n.d.	93.4	n.d.	31.1
6.3	n.d.	60.6	32.4	14.8
3.1	n.d.	24.5	6.2	0
1.6	0	0	0	0

n.d. = not defined (when growth inhibition is 50 % or more)

Table 4. Relative estrogenic activity (REA, %) of B influent and B effluent

	Before deconjugation			
concentrate (-x)	B-influent-MeOH	B-influent-EtAc	B-effluent-MeOH	B-effluent-EtAc
12.5	n.d.	0	0	0
6.3	n.d.	0	0	0
3.1	0	0	0	0
1.6	0	0	0	0
	After deconjugation			
concentrate (-x)	B-influent-MeOH	B-influent-EtAc	B-effluent-MeOH	B-effluent-EtAc
12.5	37.5	46.1	0	47.6
6.3	26.7	15.3	0	28.8
3.1	0	0	0	0
1.6	0	0	0	0

n.d. = not defined (when growth inhibition is 50 % or more)