



# Screening for estrogen active nonylphenols in surface waters by planar solid phase extraction–planar yeast estrogen screen

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## Abstract

Nonylphenols (NP) are ubiquitous in the environment and show toxic effects and estrogenic activity. According to the corresponding framework directive in the field of water quality, isomers of NP (including 4-*n*-NP and branched 4-NP) are classified as priority hazardous substances and are regulated as a group of chain and ring isomers with a maximum acceptable concentration of 2 µg/L in inland and other surface waters. This study presents a new sensitive and innovative screening approach for estrogen active NP based on high-performance thin-layer chromatography. NP were focused in a single target zone on thin-layer plates by planar solid phase extraction (pSPE) and detected by a planar yeast estrogen screen (pYES) on the basis of their estrogenic activity. The mean limits of detection and quantitation were 14 and 26 ng per zone, respectively. After liquid–liquid extraction of water samples with dichloromethane, the mean recovery was close to 100% (relative standard deviation of 21% or less), and estrogen active NP were detectable down to 1 µg/L. Thus, pSPE–pYES provides both the detection and the quantitation of estrogenic NP in surface waters at the maximum acceptable concentration. Application of the approach on extracts of surface waters showed the use of pSPE–pYES for environmental samples, and no complex and time-consuming clean-up of the extracts was required. Estrogenic NP were not detectable in any of the investigated surface waters by means of the screening approach presented.

**Keywords** Nonylphenols · Estrogen active compounds · Surface waters · High-performance thin-layer chromatography · Planar solid phase extraction · Planar yeast estrogen screen

## Introduction

Nonylphenols (NP) are substances that are ubiquitously present in our environment (e.g., surface and drinking waters), food, and drinks [1–3], and they have also been found in foodstuffs for babies and toddlers [4]. Moreover, NP were reported to be present in human blood and breast milk, and transplacental exposure of fetuses was also reported [5, 6]. NP occurring in the environment are mainly degradation products of nonylphenol ethoxylates (NPE), which are nonionic

surfactants used, for instance, in cleaning agents, and thus presumably end up in the aquatic environment [7]. Studies on the toxicity and estrogenicity of NP as environmentally relevant alkylphenols were performed, and estrogenic activity [8], toxic effects on fish [9], and reproductive problems in Japanese quails [10] were reported. Moreover, the dependency of estrogenicity on both the branching degree of the alkyl chain and the position and the length of the alkyl chain was revealed [11] when different isomers showed differing estrogenic potencies [12].

In respect to water quality, a European framework directive was established with the objective to reduce and cease, respectively, the production and the discharge into the aquatic environment of so-called priority substances and priority hazardous substances [13–16]. Priority substances are substances that “present a significant risk to or via the aquatic environment,” and the classification as priority hazardous substance additionally takes into account international agreements or legislations in terms of substances of concern [13]. Priority substances, including NP comprising isomers of 4-*n*-NP and

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This approach won the ABC poster award for excellent presentation of particularly significant innovative analytical research at ISEAC40 (International Conference on Environmental & Food Monitoring) 2018 in Santiago de Compostela, Spain.

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branched 4-NP (technical grade mixture of ring and chain isomers), plus environmental quality standards for inland and other surface waters are listed in the annexes of Directive 2013/39/EU [16]. Moreover, NP are classified as priority hazardous substances, and their environmental quality standard is stated with a maximum acceptable concentration of 2 µg/L [16].

Besides regulations concerning water quality, the general class of NP and also NPE have been regulated since 2012 by the REACH (“Registration, Evaluation, Authorisation and Restriction of Chemicals”) regulations, restricting the placing on the market or use for given purposes to 0.1% by weight [17]. Since NPE are still widely used in the manufacture of textiles in non-EU countries, possibly being washed out and leading to contamination of the environment [18], textiles containing 0.01% or more NPE by weight should not be placed on the market from February 2021 [19].

Because of existing regulations concerning NP, their ubiquitous presence, and their toxicity and estrogenicity, their determination is of great interest. Analysis is usually performed by gas chromatography–mass spectrometry or high-performance liquid chromatography with mass-selective or fluorescence detection [3, 20–23]. All reported methods require clean-up steps to prevent interference by matrix components, and/or NP have to be subjected to derivatization. Furthermore, most studies are performed as targeted analysis of certain single isomers and enantiomers of NP. As NP are regulated as the total in existing regulations regarding water quality, however, a meaningful and valuable approach for their determination is their analysis as the sum. Therefore, a simple and sensitive screening method for total NP was developed, and detection was based on their estrogenicity. The combination of the planar solid phase extraction (pSPE) concept [24] and the planar yeast estrogen screen (pYES) [25], which are both based on high-performance thin-layer chromatography (HPTLC), is the optimal method. NP are focused by pSPE on HPTLC plates in a single target zone, and simultaneously clean-up is realized, thus providing the analysis of matrix-rich sample extracts without time-consuming and complex clean-up. Subsequently, the detection of NP aside from natural and synthetic estrogens is done on the basis of their estrogenic activity by a pYES.

## Materials and methods

### Chemicals and materials

Agar, adenine, casamino acids [26], and yeast nitrogen base without amino acids (YNB) for agar plates were from Becton, Dickinson (Heidelberg, Germany), and YNB for culture media was purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (>99.9%), ethyl acetate (for pesticide

residue analysis), L-lysine ( $\geq 98\%$ ), magnesium sulfate heptahydrate (puriss. p.a.), and toluene (for pesticide residue analysis) were from Fluka-Sigma-Aldrich (Steinheim, Germany), and Chemsolute *n*-hexane (for pesticide residue analysis) was from Th. Geyer (Renningen, Germany). Sodium sulfate (anhydrous,  $\geq 99.0\%$ ), ethanol ( $\geq 99.8\%$ ), 17 $\beta$ -estradiol (E2;  $\geq 98\%$ ), and 17 $\alpha$ -ethinylestradiol (EE2;  $\geq 98\%$ ) were purchased from Sigma-Aldrich. Copper(II) sulfate pentahydrate (p.a.), disodium hydrogen phosphate (p.a.), hydrochloric acid (37%, for analysis), L-histidine ( $\geq 99\%$ ), potassium chloride (>99%), potassium dihydrogen phosphate (p.a.), sodium hydroxide solution (20%), and glass HPTLC silica gel 60 RP-18 W plates (20 cm  $\times$  10 cm) were from Merck (Darmstadt, Germany). BDH Prolabo D-glucose (anhydrous, >99%) and sodium hydrogen carbonate (100%) were purchased from VWR International (Bruchsal, Germany). Resorufin- $\beta$ -D-galactopyranoside (RGP) was from Santa Cruz Biotechnology (Heidelberg, Germany), dimethyl sulfoxide (99.98%) was from Fisher Scientific (Schwerte, Germany), and acetone ( $\geq 99.8\%$ ) and dichloromethane (>99.5%) were from Carl Roth (Karlsruhe, Germany). 4-NP NP1 (99%; mixture of isomers) was from Acros Organics (Thermo Fisher Scientific, Geel, Belgium), 4-NP NP2 (mixture of branched chain isomers) from was TCI Europe (Eschborn, Germany), 4-*n*-NP NP3 (98%+) was from Alfa Aesar (Karlsruhe, Germany), 4-NP NP4 (mixture of compounds with branched side chains) was from abcr (Karlsruhe, Germany), and NP NP5 (Pestanal, analytical standard, technical mixture) and NP NP6 (technical grade, mixture of ring and chain isomers) were from Sigma-Aldrich. Ultrapure water was produced by a Synergy system (Millipore, Schwalbach, Germany). Yeast cells of the recombinant strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MAT $\alpha$ , PEP4::HIS3, prb1- $\Delta$ 1.6R, HIS3- $\Delta$ 200, lys2-801, trp1- $\Delta$ 101, ura3-52gal2can1) were generated by McDonnell et al. [27, 28] and stored in a cryovial at -70 °C. Orange filter glass O 580 from HEBO (Aalen, Germany) was cut to shape as an optical filter for a TLC Scanner 4 (CAMAG, Muttenz, Switzerland).

### Stock and standard solutions

Stock and standard solutions were prepared in ethanol and stored at -20 °C. Standard stock solutions of NP1–NP6 were prepared at a concentration of 2 g/L. Standard solutions of NP1–NP6 were obtained by dilution of the stock solutions to a concentration of 50 mg/L and were used to investigate the responses of NP1–NP6 to the pYES. The standard solution of NP4 was further diluted to concentrations of 10 and 5 mg/L for spiking and calibration, respectively. Standard stock solutions of E2 and EE2 were prepared at a concentration of 1 mg/L and were diluted for application (100 µg/L).

## Media, agars, and cultivation of yeast cells

Growth medium for overnight cultures was prepared as described in earlier work (YNB at 6.8 g/L, glucose at 1 g/L, L-lysine at 170 mg/L, and L-histidine at 100 mg/L), whereas the solution of glucose and the solution containing YNB plus amino acids were autoclaved by steam separately and mixed afterward [25]. To obtain the test medium for yeast incubation of HPTLC plates, additionally  $\text{CuSO}_4$  (112  $\mu\text{M}$ ) was added to the growth medium [25]. Plate agar was composed of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L), and agar (20 g/L) [25]. Yeast agar plates with single colonies were obtained by streaking cell mass in a three-phase streaking pattern on the agar plate and subsequent incubation of the plate for at least 2 days at 30 °C [25]. Yeast suspensions were prepared by inoculation of growth medium with a colony from the yeast agar plate and shaking overnight at 30 °C (150 rpm, incubating minishaker, orbit 3 mm, VWR International, Darmstadt, Germany). To prepare test cultures with a cell number of  $6 \times 10^7$ – $8 \times 10^7/\text{mL}$  [25], the required amount of overnight cultures was centrifuged and cell pellets were resuspended in the test medium. Cell numbers were measured with a TC20 automated cell counter (Bio-Rad Laboratories, Munich, Germany).

## Substrate solution

The dipping solution for substrate incubation of the HPTLC plates was prepared by dilution of 200  $\mu\text{L}$  of a stock solution of RGP (20 mg/mL in dimethyl sulfoxide, stored at -20 °C) with 40 mL of phosphate buffer ( $\text{KH}_2\text{PO}_4$  at 40.8 g/L,  $\text{Na}_2\text{HPO}_4$  at 42.6 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  at 1.2 g/L, and KCl at 3.7 g/L, adjusted to pH 7 with sodium hydroxide solution) [25].

## Planar solid phase extraction–planar yeast estrogen screen

Glass HPTLC silica gel 60 RP-18 W plates were prewashed and pretreated according to [25]. In brief, the plates were prewashed with acetone/water (9:1, v/v), dried at 120 °C for 30 min on a TLC Plate Heater (CAMAG), immersed in a solution of sodium hydrogen carbonate (25 g/L, adjusted to pH 6.4 with hydrochloric acid) with use of a TLC Chromatogram Immersion Device III (CAMAG, time 3 s, speed 3) for pH adjustment of the layer, and dried in a cold airstream for 5 min. Pretreated plates were stored in a desiccator until use. Standard and sample solutions were applied onto the plates (cut to 10 cm  $\times$  10 cm) with an Automatic TLC Sampler 4 (CAMAG) at 10 mm from the lower edge and 12 mm from the left side, and ethanol was used as the rinsing solvent. Extracts of water samples (15  $\mu\text{L}$ ) were applied as 5 mm  $\times$  10 mm areas and standard solutions were applied as 5-

mm bands (track distance set to automatic). To focus application areas before pSPE, the plate was developed with acetonitrile up to 15 mm and dried for 2 min in a cold airstream. For pSPE, twofold development in a twin-trough chamber (10 cm  $\times$  10 cm, CAMAG) was performed: the plate was developed with *n*-hexane/acetonitrile/toluene (4:2:1.5, v/v/v) up to a migration distance of 60 mm and dried for 2 min in a cold airstream, followed by the second development with *n*-hexane/ethyl acetate/toluene (2.5:4:0.5, v/v/v) up to 50 mm. After the plate had been dried in a cold airstream for 5 min, the detection of estrogen active analytes by the pYES followed according to [25]. For this purpose, the plate was immersed in a suspension of genetically modified yeast cells adjusted to  $6 \times 10^7$ – $8 \times 10^7$  cells per milliliter (time 3 s, speed 3), left in the vertical position for 30–60 s for homogeneous distribution of yeast cells, and incubated in a closed box equipped with filter paper and water (relative humidity approximately 100%, 30 °C) for 4 h. The dried plate (5 min, cold airstream) was then immersed in the substrate solution (0.1 mg RGP per milliliter, time 3 s, speed 3) and again incubated in a closed box (relative humidity approximately 100%, 37 °C) for 30 min. Drying, dipping, and incubation with RGP was repeated two times [25]. Documentation of the plate was done under 254 nm UV light (TLC Visualizer, CAMAG), and densitometry was performed with a TLC Scanner 4 in fluorescence mode at 550 nm > 580 nm using the tungsten lamp. All HPTLC instruments were controlled by winCATS, version 1.4.6 (CAMAG).

## Limits of detection and quantitation

To determine the limit of detection (LOD) and the limit of quantitation (LOQ), a standard solution of NP4 (5 mg/L) was applied in different volumes (1–10  $\mu\text{L}$ ) onto HPTLC plates ( $n = 8$ ), resulting in amounts of 5–50 ng NP4 per zone. Subsequently, pSPE–pYES was performed as described earlier, and the LOD and LOQ were determined by the signal-to-noise ratio (peak height).

## Extraction of water and recovery rate

Extraction of water by liquid–liquid extraction (LLE) was evaluated by testing two extraction solvents and methods (*n*-hexane versus dichloromethane, and stirring versus shaking; each for 10 min and  $n = 6$ ). LLE finally was performed with dichloromethane by stirring. Ultrapure water was spiked with NP4 at a concentration of 2  $\mu\text{g}/\text{L}$ , and an aliquot of 200 mL was extracted with 20 mL of dichloromethane by stirring on a magnetic stirrer (MR3001, Heidolph Instruments, Schwabach, Germany) for 10 min at 1000 rpm. The separated organic phase was evaporated to dryness under a gentle stream of nitrogen, and the residue was dissolved in 200  $\mu\text{L}$  of ethanol, resulting in a concentration factor of 1000. Extracts were stored at -20 °C until analysis. Blank extracts (extraction of

ultrapure water) and blank tracks (application of absolute ethanol) served as controls. The pSPE–pYES of extracts and controls was performed as described earlier with a five-point calibration (15–40 ng NP4 per zone) and a new yeast test culture each time ( $n = 3$ ).

### Analysis of surface water

As environmental samples, water samples were taken from a stream course and a pond in a public park (Hohenheim, Stuttgart, Germany, February 2019), from Lake Constance (October 2018), and from the Kleinhohenheimer Bach (two samples plus one of a small influent stream) and the Körsch (tributaries of the river Neckar, Stuttgart, Germany, February/March 2019). Aliquots (200 mL) of the water samples were extracted with dichloromethane as explained earlier (each  $n = 1$ ); however, in the case of emulsions occurring after stirring, the emulsion was separated together with the organic phase from the water phase, and sodium sulfate was added to break the emulsion before evaporation of the clear organic phase. Extracts were stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. The extracts were applied next to a blank track and a five-point calibration (15–40 ng NP4 per zone) and analyzed by pSPE–pYES as described earlier. Additionally, 200 mL of each environmental sample were spiked with NP4 at a concentration of  $2\text{ }\mu\text{g/L}$ , extracted, and investigated in the same way as the native samples (each  $n = 1$ ).

### Determination of the E2-equivalent factor

A standard solution of NP4 (5 mg/L) was applied in different volumes (1–10  $\mu\text{L}$ ) and pSPE–pYES was performed as described earlier ( $n = 8$ ). The half-maximal effective dose ( $\text{ED}_{50}$ ) of NP was determined with OriginPro version 2018b (OriginLab, Northampton, MA, USA) from the fitted sigmoidal curve obtained by plotting the peak heights of the signals against the decadic logarithm of the applied amounts per zone as described in previous work [29]. The E2-equivalent factor (EEF) of NP4 was calculated as follows:

$$\text{EEF (NP4)} = \frac{\text{ED}_{50} (\text{E2})}{\text{ED}_{50} (\text{NP4})}. \quad (1)$$

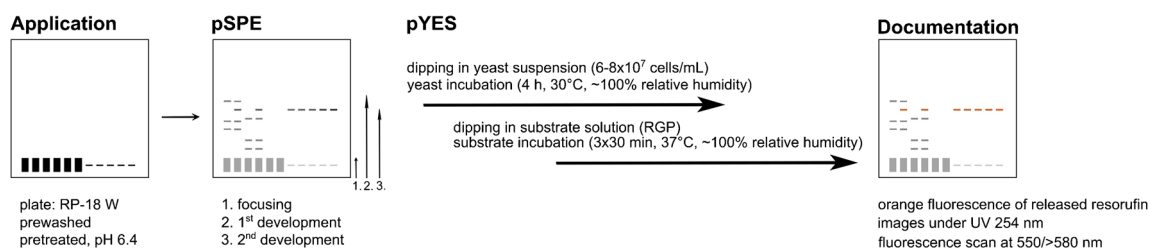
## Results and discussion

### pSPE–pYES for the detection of estrogen active NP

NP usually occur not as a specific compound but as complex mixtures of isomers and enantiomers [7]. They are ubiquitous substances that show estrogenic activity and thus are a major concern regarding the aquatic environment. Because

of this and because of existing regulations concerning water quality, the estrogenicity of NP is the main focus of this study and was used for their detection as the total instead of analyzing individual isomers. The pYES provides the detection and determination of estrogen active compounds on planar thin-layers [25] by means of genetically modified yeast cells containing the human estrogen receptor and a reporter gene encoding  $\beta$ -galactosidase [27, 28]. In the presence of estrogenic substances, the enzyme is expressed and cleaves the substrate RGP, releasing orange fluorescing resorufin as positive signal of estrogenicity in the respective zones. Combined with the concept of pSPE, which separates analytes of one or more chemical groups from the matrix and other compounds and simultaneously focuses the analytes in one or more target zones [24, 30, 31], estrogen active NP can be detected on the basis of their estrogenicity by pSPE–pYES as the sum (for the work flow, see Fig. 1). Development of pSPE was done according to previous studies (data not shown) [24, 30, 31] with the aim of focusing NP in a common zone. The twofold development in the same direction served to separate some matrix compounds and additionally natural and synthetic hormones after the first development and to subsequently focus broadened zones of NP into a sharp target zone with the second development. Thus, estrogen active NP were easily detectable by means of the pYES as the sum (exemplarily shown in Fig. 2 for different technical mixtures of NP, the natural hormone E2, and the synthetic hormone EE2), matching existing regulations (Water Framework Directive [13–16]). NP showing estrogenicity were therefore ascertained as the total all at once without the requirement of any assessment of individual isomers. Use of the pYES as a detection method provides the great advantage of the high selectivity of this screen. Clear positive signals, indicated by orange fluorescence of released resorufin, are based only on yeast and reporter gene activity after estrogen active compounds have been bound by the human estrogen receptor. Thus, no false positive results or interferences by possibly co-migrating, nonestrogenic compounds are to be expected. Since the screening is based on pSPE, which simultaneously provides a planar clean-up, as already shown in previous studies for different matrices [24, 25, 30–32], the pSPE–pYES approach is suitable for the analysis of matrix-rich extracts, omitting complex sample treatments. Contrarily, analysis of NP by gas chromatography–mass spectrometry, for example, requires clean-up of the extracts by high-performance liquid chromatography [3, 20] or solid phase extraction [21], and NP have to be subjected to derivatization. Additionally, NP are often analyzed as targeted individual isomers. The opportunity to apply matrix-rich extracts directly to the analysis followed by the detection of NP as the total, therefore, is another advantage of pSPE–pYES. As an HPTLC method, moreover, the screening





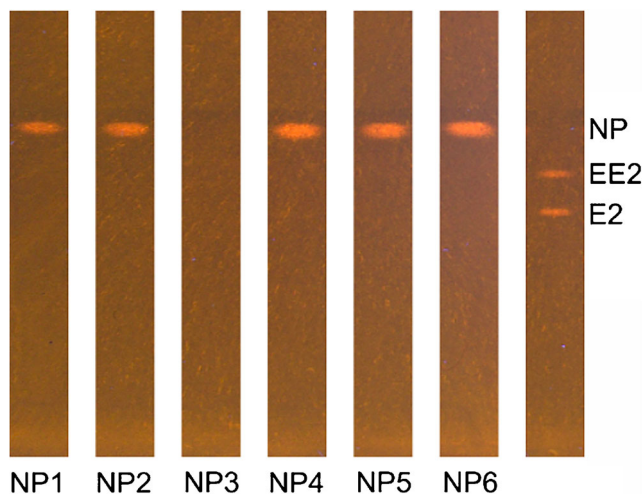
**Fig. 1** Work flow of planar solid phase extraction (pSPE) combined with the planar yeast estrogen screen (pYES). After application and pSPE, a pYES is performed by dipping the high-performance thin-layer chromatography plate into yeast suspension and substrate solution (resorufin- $\beta$ -

D-galactopyranoside, RGP), respectively, followed by the corresponding incubations and the final documentation (for details, see “[Materials and methods](#)”)

provides the analysis of at least 15 samples in addition to a multipoint calibration in a single run when 20 cm  $\times$  10 cm plates are used. Therefore, the pSPE–pYES developed is a very innovative, selective, straightforward, high-throughput and targeted screen for the group of environmentally relevant and estrogen active NP.

### Signal response of different NP

First, the response of six purchased NP (NP1–NP6) to pSPE–pYES was evaluated after application of 200 ng per track. NP3, with a linear nonyl side chain, did not show any remarkable response to the pYES at the amount investigated, whereas the technical mixture NP4 showed the highest response (Fig. 2). The other technical mixtures of NP tested (NP1, NP2, NP5, NP6) gave an average response to the pYES of 80% (61–97%) as compared with NP4. Thus, the technical mixture NP4 was used as a representative reference with the highest response of the group of estrogenic NP.



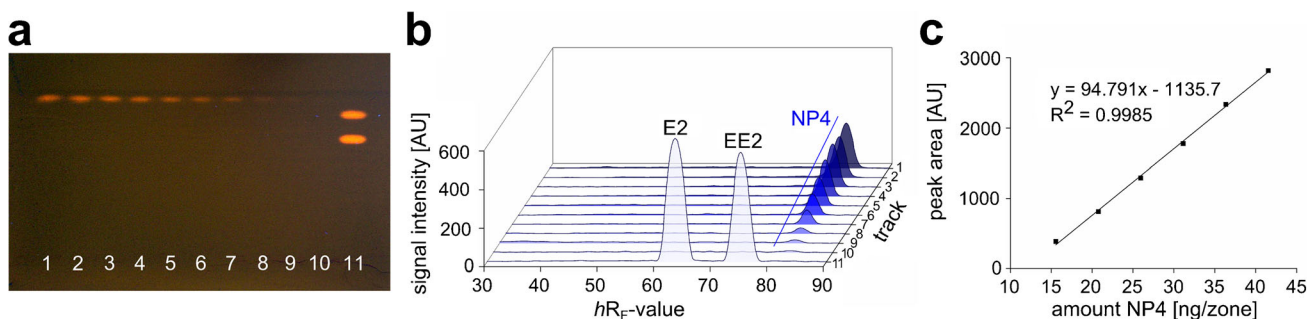
**Fig. 2** Images of tracks of different nonylphenols (NP; each 200 ng per zone) after planar solid phase extraction–planar yeast estrogen screen under 254 nm UV illumination and a track with 17 $\alpha$ -ethinyloestradiol (EE2) and 17 $\beta$ -estradiol (E2) as examples of synthetic and natural hormones (each 200 pg per zone). NP1, NP2, NP4, NP5 and NP6 are technical mixtures of NP; NP3 is 4-*n*-NP

### Sensitivity and working range

The LOD and LOQ were determined by the signal-to-noise ratio after the application of different amounts of the reference NP4 (5–50 ng NP4 per zone), where a signal-to-noise ratio of 3 or greater defines the LOD and signal-to-noise ratio of 10 or greater defines the LOQ [33]. An image of an HPTLC plate after pSPE–pYES under 254 nm UV illumination and the respective 3D densitogram of the fluorescence scan at 550 nm/> 580 nm is exemplarily shown in Fig. 3. On each plate the amount of NP with a response of at least three times and ten times the noise signal (highest noise of the plate), respectively, was determined. The mean LOD and LOQ were determined to be  $14 \pm 4$  ng per zone and  $26 \pm 4$  ng per zone ( $n = 8$ ), respectively. The working range for quantitation of NP was chosen on the basis of the LOD ascertained and the correlation of linear calibration graphs (peak area plotted against amount per zone) of several applications. Calibration graphs in a working range of 15–40 ng per zone showed adequate linearity (Fig. 3c): the coefficients of determination ( $R^2$ ) were between 0.9561 and 0.9985 (mean of 0.9788,  $n = 8$ ).

### Sample extraction and recovery rates

A simple LLE was chosen for the extraction of water samples since pSPE–pYES offers a robust planar screening, designed to provide reliable results from matrix-containing extracts. Initially, two extraction solvents (dichloromethane and *n*-hexane) and methods (stirring and shaking, each for 10 min) for the extraction of water samples were tested and compared. Water was spiked with NP4 at 2  $\mu$ g/L, representing the maximum acceptable concentration in inland and other surface waters, and aliquots (200 mL) were extracted (20 mL extraction solvent) in conical flasks by stirring on a magnetic stirrer or shaking on an orbital shaker. Extracts were applied after concentration (factor 1000) with an application volume of 15  $\mu$ L, leading to an amount of 30 ng per zone (above the LOQ) on the supposition of a complete extraction. Recoveries were determined by applying the extracts next to three standard levels and comparing the results.



**Fig. 3** **a** Image of a high-performance thin-layer chromatography plate for the determination of the limits of detection and quantitation after planar solid phase extraction–planar yeast estrogen screen under 254 nm UV illumination. **b** Corresponding 3D densitogram of the

fluorescence scan at 550 nm/> 580 nm. **c** Calibration graph of the plate shown in the range from 15 to 40 ng per zone. Tracks 1–10 are for 5–50 ng NP4 per zone; track 11 is for 200 pg 17 $\alpha$ -ethinylestradiol (EE2) and 17 $\beta$ -estradiol (E2) per zone, respectively

Extraction with dichloromethane by stirring provided the highest recoveries, and the lowest relative standard deviation (RSD) was additionally obtained (data not shown). Therefore, dichloromethane and stirring were used for LLE for recovery experiments and extraction of environmental water samples. Recovery experiments for the final extraction method were performed on three different days with a new test culture and HPTLC plate each time. The experiments were successful, with a mean recovery rate of  $(95 \pm 17)\%$  for NP4 from spiked water samples ( $n = 3$  days each with  $n = 4$  extracts), and very repeatable, with an intraday precision expressed as the RSD of 21% or less (Table 1,  $n = 4$ ) and an interday precision of 18% or less ( $n = 3$ ). With regard to the extraction method and the sensitivity of pSPE–pYES, the approach developed allowed the detection of estrogenic NP in water down to about 1  $\mu\text{g/L}$  (LOD  $0.9 \pm 0.3 \mu\text{g/L}$ ). From the achieved working range of trace analysis and from the dependency of the outcome of the screening on the daily condition and number of yeast cells, the high recovery rates and precision demonstrate good accuracy of the simple LLE combined with pSPE–pYES. Thus, the applied LLE coupled with pSPE–pYES is a suitable approach to screen samples for estrogen active NP at environmentally relevant concentrations in surface waters.

With regard to extraction and application and the response of NP1, NP2, NP5, and NP6 (61–97% of the NP4 response), the screening provides clear detection of estrogenic NP in water samples above the LOD and in the range of the LOQ. The application volume of the extracts can also be increased to obtain a lower LOD or LOQ if necessary. The stated aspects are, however, invalid for NP3, with a linear side chain (4- $n$ -NP), because no estrogenic activity was observed by the pYES at the tested and relevant amounts.

### Screening of surface waters

Samples of surface waters from different public sites (see “Materials and methods”) were extracted and analyzed by

pSPE–pYES. In none of the samples investigated were estrogenic NP or other estrogenic compounds detected. The application of pSPE–pYES for surface waters without time-consuming extraction and purification, however, showed the great advantage of the screening approach for environmental, matrix-rich samples. To prove the performance of the screening for the detection of NP from surface water, the environmental samples were additionally spiked with NP4 at a concentration of 2  $\mu\text{g/L}$ , extracted, and subjected to pSPE–pYES. Estrogenic NP were detectable as indicated by orange fluorescence of resorufin as a positive signal of estrogenicity aside from many other native fluorescing components, whereas most of the co-extracted matrix compounds remained in the application area (Fig. 4). A few others were distributed over the migration distance (visible as native fluorescing zones showing red and blue fluorescence, respectively) but did not affect the detection of the orange fluorescence generated by estrogenic analytes. Compared with the recoveries from ultrapure water, the extraction efficiency for NP4 from surface waters was in the same range, with the recovery rate determined to be  $(95 \pm 11)\%$  ( $n = 7$ ). Analyses of spiked environmental samples showed the good applicability of the combination of LLE and pSPE–pYES for surface waters. The good accuracy and repeatability of the method were again shown by a high recovery rate and a good precision of 11% or less ( $n = 7$ ).

### EEF of 4-NP

The EEF of estrogen active NP was determined by the application of different volumes of a solution of the representative NP4 (5–50 ng per zone), followed by pSPE–pYES. By plotting the peak heights of the signals against the respective logarithmic amounts per zone, sigmoidal dose–response curves were obtained (exemplarily shown in Fig. 5), and the  $\text{ED}_{50}$  values given by the inflection point of the curves were calculated with OriginPro.  $\text{ED}_{50}$  was determined to be  $29 \pm 6$  ng per zone ( $n = 8$ ). By

**Table 1** Recovery rates for NP4 from spiked water samples (each  $n = 4$ )

Day	Mean recovery $\pm$ RSD (%)	Coefficient of determination of calibration ( $R^2$ )
1	115 $\pm$ 7	0.9749
2	96 $\pm$ 21	0.9898
3	74 $\pm$ 6	0.9959

Repetitions were performed on 3 days

RSD relative standard deviation

means of  $ED_{50}$  of NP4 and  $ED_{50}$  of E2, the estrogenic potency of NP4 relative to E2 was determined, expressed as the EEF (Eq. 1). Thus, with  $ED_{50}$  of 29 ng per zone for NP4 and  $ED_{50}$  of 47 pg per zone for E2 [29], the EEF and thus the estrogenic potential of the technical mixture NP4 was calculated to be 0.0016.

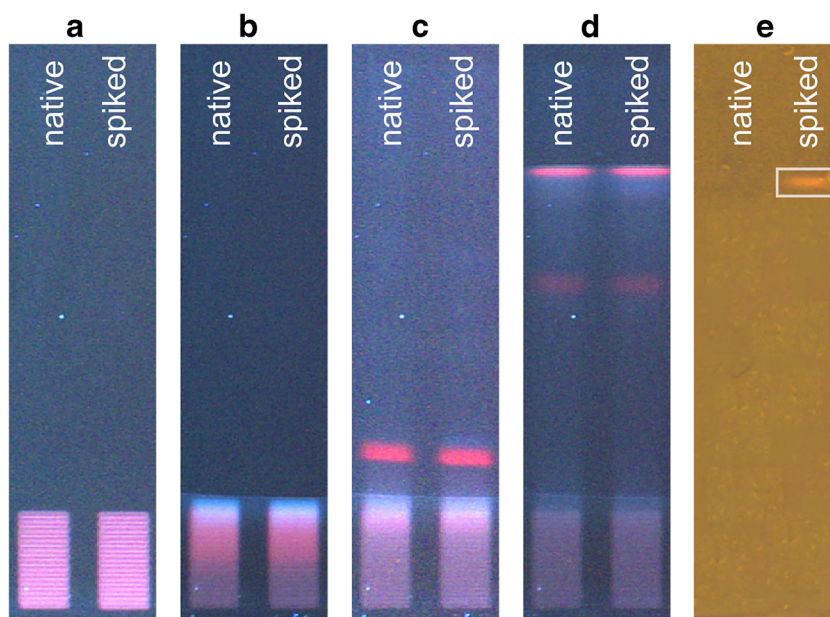
The determination of the EEF by pSPE–pYES was performed with a representative reference technical mixture of 4-NP (NP4). From responses between 61% and 97% for the other technical mixtures investigated (NP1, NP2, NP5, NP6), calculated on the basis of the response of NP4, they showed estrogenic activity relative to E2 of the same order of magnitude. However, NP3 (4-*n*-NP) did not show any response at the amounts tested compared with the technical mixtures consisting of ring and chain isomers of NP (NP1, NP2, NP4, NP5, NP6) (Fig. 2). In the literature, very different data for the estrogenic potential of NP, obtained by various assays, are reported [34–36]. The estrogenic potency determined by microtiter plate yeast estrogen screen assays ranged from  $7.2 \times 10^{-7}$  to 0.0011 [35, 36], the latter well matching the EEF determined in the present work. In a study using a similar pYES,

the respective EEF was determined to be  $2.3 \times 10^{-4}$  for 4-*n*-NP [34], thus for 4-NP with a linear side chain, which could be the reason for the deviation.

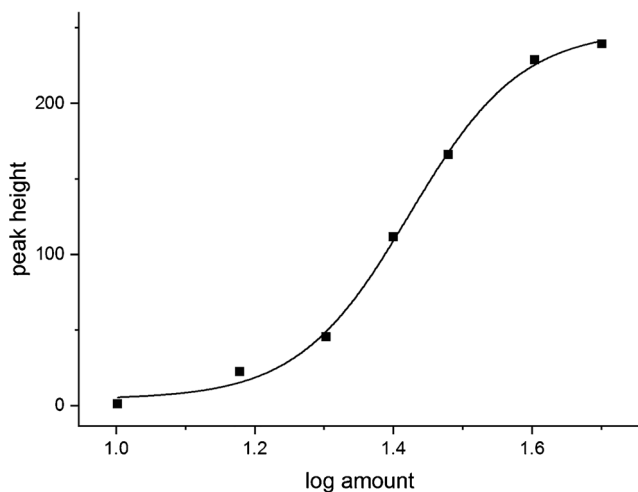
### Prospects and options

The screening presented provides reliable, sensitive, and selective detection of NP showing estrogenic activity. Additionally, the approach generally can provide further information, since all NP will be focused in the target zone. Therefore, it offers the option to further analyze all NP present in a sample by mass spectrometry after elution of the substances in the target zone with a thin-layer chromatography–mass spectrometry interface directly coupled to a mass spectrometer or after elution into autosampler vials. Thus, a second dimension of chromatography can be applied (e.g., by liquid chromatography or gas chromatography), also with possibly required derivatization, which, however, was not the aim of the present work. Moreover, HPTLC analysis generally offers the possibility to apply higher volumes or amounts of analytes, if required, without falsifying or negatively affecting the outcome of the screening, whereby the detection of estrogenic NP with lower responses to the pYES can also be easily realized. Furthermore, with the pYES, the estimation of the estrogenic potency of samples caused by single substances is possible, as shown for hop samples in previous work [32]. Differing volumes of a sample extract can be applied and dose–response curves can be plotted from the signals, from which the half-maximal effective volume can be determined, which is subsequently used to calculate the estrogenic potency relative to E2 [29, 32]. Therefore, the total estrogenic potency of water samples can be estimated, including the potency

**Fig. 4** Images of a high-performance thin-layer chromatography plate of extracts of a native and spiked (NP4 at 2  $\mu$ g/L) sample of surface water from a pond in a park under 366 nm UV illumination after application of 15  $\mu$ L (a), after a focusing step with acetonitrile (b), after planar solid phase extraction (twofold development; c, d), and under 254 nm UV illumination after the planar yeast estrogen screen (e)







**Fig. 5** Dose–response curve for nonylphenols (technical mixture) for the determination of the half-maximal effective dose, shown for an application range of 10–50 ng per zone for the reference NP4

caused by NP and simultaneously the estrogenic potency caused by other known or unknown estrogen active compounds in the sample. Finally, pSPE was introduced earlier as reliable approach for the clean-up of extracts of complex matrices as shown for fruits and vegetables [24], rye flour [30], and paper and cardboard [30]. Because of this clean-up effect, the screening developed ought to be a suitable method for the detection of estrogen active NP in extracts of more complex matrices such as food.

## Conclusions

The pSPE–pYES approach developed was shown to be a highly sensitive and very effective tool for the screening for estrogen active NP in water samples after suitable extraction, and the total NP are easily determined below the existing maximum acceptable level of 2 µg/L. NP were extractable with high recoveries by LLE from both spiked ultrapure and surface water samples with a precision as expressed by the RSD of 21% or less. As a screening tool, pSPE–pYES offers the simultaneous analysis of several samples, and provides the detection of the estrogenic activity caused by all NP isomers focused in a single target zone. Hence, the risk of samples on the basis of the estrogenicity of NP is ascertained collectively at once, and no risk assessment in terms of the estrogenicity of individual isomers is required. The use of the planar chromatographic pSPE approach offers both the advantage of reduced sample preparation since the layer provides a planar clean-up and the advantage of focusing the group of NP in a sharp zone. Therefore, the detection of the total estrogenic compounds is possible aside from co-extracted matrix substances, with no need for complex sample preparations. Furthermore, because of the orange fluorescence of resorufin

released from the substrate as a positive signal of estrogenicity during the pYES, the targeted estrogenic analytes are selectively detected and easily distinguishable from matrix components showing native fluorescences.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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