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Determination of the xenoestrogens 4-nonylphenol and bisphenol A by high-performance liquid chromatography and fluorescence detection after derivatisation with dansyl chloride

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Abstract

An easily performable and highly selective method for the determination of the xenoestrogens bisphenol A (BPA) and technical 4-nonylphenol (mixture of isomers) from environmental samples was developed. The method consists of fluorigenic labelling of the substances by dansylation followed by HPLC separation of the derivatives. Specific wavelengths (λ_{ex}=354 nm, λ_{em}=545 nm) for detection of the dansylated phenols were determined in order to reduce the signals of interfering compounds. The applicablility of the method for environmental samples was demonstrated by using sewage sludge spiked with BPA and 4-*n*-nonylphenol (as internal standard).

Keywords: Nonylphenols; Phenols; Bisphenol A; Xenoestrogens

1. Introduction

Xenoestrogens are synthetic chemicals with widely diverse chemical structures which can bind to estrogen receptors and therefore mimic the action of estrogens. In recent years, interest concerning xenoestrogens in the environment has increased because several studies have shown the appearance of xenoestrogens in environmental compartments correlated with malformations and reduced fecundity in human and wildlife [1–4].

The widely utilized industrial chemicals 4-nonylphenol (4-NP) and bisphenol A (BPA) (Fig. 1) belong to the group of xenoestrogens [5]. They show estrogenic effects in fish, avian and mammalian cells [1,6]. It should be remarked that technical 4-NP is a mixture of isomers with regard to branching of the alkyl chain. The composition of technical 4-NP is not known in detail; however, the product does not contain 4-*n*-nonylphenol (4-n-NP) [7].

During experiments concerning the elimination of 4-NP and BPA from wastewater, the need for a sensitive, easily performable method for the quantitative determination of BPA and technical 4-NP from environmental samples (e.g. sewage sludge) and the linear isomer 4-n-NP (as internal standard) has emerged. It was required to determine all three

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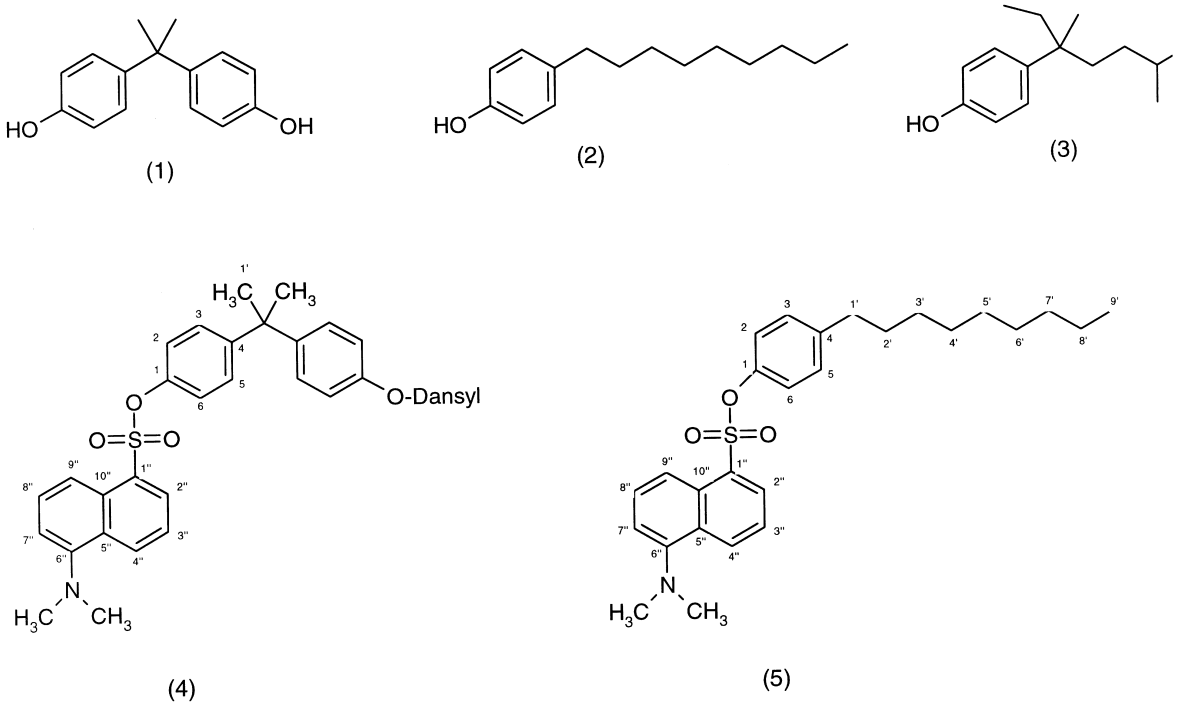

Fig. 1. Chemical structures of bisphenol A (1), 4-*n*-nonylphenol (2), one branched isomer of technical 4-nonylphenol (3) and the dansyl derivatives of bisphenol A (4) and 4-*n*-nonylphenol (5).

substances from one sample. In order to quantify the total amount of technical 4-NP it was necessary to avoid separation into its different isomers. On these criteria, it has been decided to use high-performance liquid chromatography (HPLC) rather than gas chromatography (GC) which leads to a complex peak pattern for technical 4-NP [7,8].

The xenoestrogens 4-NP and BPA can be detected by their natural fluorescence [9,10]. We found a considerable interference of the xenoestrogens with other compounds in sewage sludge when measured at wavelenghts often used for detection of nonylphenol and bisphenol A (λ_{ex}=277 nm, λ_{em}=300 nm; [10,11]). In order to enhance the specificity of determination the samples were derivatised with the fluorigenic reagent dansyl chloride. Dansyl chloride is known to react easily with phenolic hydroxyl and amino groups and therefore reacts only with certain components in environmental samples. The fluorophores formed by dansylation possess specific fluorescence properties determined in this work (see Section 2.5). The interference of the xenoestrogens with other compounds decreases when detection is performed at wavelenghts specific for the dansyl derivatives.

The dansylation of some phenolic compounds with dansyl chloride has been reported before [12,13]; so far as we found, the reagent has not been used for the quantitative determination of 4-NP and BPA.

2. Materials and methods

2.1. Chemicals and solvents

4-n-NP (purity >98%) was purchased from Avocado (Heysham, UK). The technical mixture of 4-NP (purity ~85%, based on the content of *p*-isomers) was supplied by Fluka (Deisenhofen, Germany), BPA (purity 99.9%) by Promochem (Wesel, Germany). 5-(Dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride, purity >98%) was obtained from Merck Eurolab GmbH (Darmstadt, Germany). Acetonitrile (Rotisolv gradient grade,

purity >99%) from Roth (Karlsruhe, Germany) and bidistilled water were used exclusively as HPLC eluents.

2.2. Standard solutions

For each BPA, technical 4-NP and 4-n-NP stock solutions were prepared by dissolving 1 mg in 1 ml acetone. Standard solutions were achieved by serial dilution (0.01 μg–1.0 μg/ml) of stock solutions with acetone.

2.3. Analytical derivatisation of phenolic compounds with dansyl chloride

For the derivatisation, 3–5 μl of an 0.5 M aqueous solution of Na_2CO_3 and 20 mg of solid dansyl chloride were added to 1–2 ml of a solution containing a variable concentration of the respective phenols (at least a 10-fold excess of dansyl chloride). The reaction was accomplished in capped 10 ml glass tubes in the dark for 16 h at room temperature. After the reaction, acetone was evaporated with a slight stream of nitrogen. Then, the remaining residue was dissolved in 1.5 ml of toluene. The toluene solution was transferred to a 25 ml round flask and the solvent was removed at 50 °C at reduced pressure (80 mbar). Subsequently, the remaining dansyl derivative was dissolved in 2 ml of acetonitrile and filtered (0.45 μm) prior to analysis.

To ensure the completeness of dansylation we compared the peak areas of diluted pure dansyl derivatives (see Section 2.4) with the theoretical areas taken from our calibrations (see Section 3.2). We found yields above 98% for all reactions.

2.4. Preparative derivatisation of 4-n-nonylphenol and bisphenol A

Generally, the synthesis was performed as described in Section 2.3. For this study, 100 mg of the phenol, 1 g of dansyl chloride and 100 μl aqueous solution of 0.5 M Na_2CO_3 were reacted in 5 ml acetone for 16 h at room temperature. After removal of acetone, the derivatives were extracted with 40 ml toluene and the organic phase was transferred to a 100 ml round flask. The solvent was removed at 50 °C and reduced pressure (80 mbar). The remaining residue was subjected to preparative TLC on silica gel (SIL G-100 UV_{254}, Macherey–Nagel, Düren, Germany) using toluene–methanol (1:1, v/v) as mobile phase. Both dansyl derivatives were unequivocally identified by their ^1H- and ^{13}C-NMR spectra (structure of the dansyl derivatives in Fig. 1).

2.5. HPLC apparatus and chromatographic conditions

The HPLC apparatus consisted of a System Gold Beckman chromatograph (Munich, Germany), equipped with a Rheodyne injector joined to a 100 μl loop, a Beckman System Gold Programmable Solvent Module 125 and a Shimadzu RF-551 spectrofluorometric detector. The chromatography was carried out on an ET 250/4 Nucleosil 100-5 C_{18} column (250×4 mm, Macherey–Nagel). Water (solvent A) and acetonitrile (solvent B) were used as mobile phase according to the following gradient program: A–B (50:50, v/v) for 5 min; linear gradient from A–B (50:50, v/v) to A–B (0:100, v/v) for 15 min; A–B (0:100, v/v) for 10 min; return to A–B (50:50, v/v) for 5 min; A–B (50:50, v/v) for 5 min. Chromatography was performed at 25 °C with a flow-rate of 1 ml/min and an injection volume of 100 μl.

Fluorescence conditions were as follows: $\lambda_{ex}=354$ and $\lambda_{em}=545$ nm. These conditions were found by recording excitation and emission spectra at several wavelengths. The recordings were performed by measuring pure derivatives dissolved in acetonitrile.

2.6. Determination of 4-n-nonylphenol and bisphenol A in sewage sludge

For the determination of 4-n-NP and BPA from sewage sludge, 100 ml of sewage sludge (activated sludge from a municipal sewage treatment plant, 0.4% dry matter) were dried by lyophilization for 16 h. Then, the sample was spiked with 10 μg of 4-n-NP and 10 μg of BPA dissolved in 10 μl of methanol. The spiked sludge was subsequently extracted with cyclohexane for 4 h in a Soxhlet apparatus. The extract was taken to dryness under a slight stream of nitrogen and the remaining residue was dissolved in 2 ml of acetone. Derivatisation was performed as described in Section 2.3. The re-

coveries were 78% for 4-n-NP and 18% for BPA (for discussion see Section 3.3).

3. Results and discussion

3.1. Chromatography

A typical HPLC chromatogram for dansylated standards of BPA, technical 4-NP and 4-n-NP is shown in Fig. 2. The derivatives of the pure compounds BPA and 4-n-NP gave sharp peaks exhibiting a width of about 0.5 min. In contrast, the derivatized technical 4-NP gave a broad peak displaying a width of about 1.7 min (see Fig. 2) caused by an incomplete separation in its isomers. For the dansyl derivatives the following retention times were found: dansyl-BPA, 24.1 min; dansyl-technical 4-NP, 25.5 min; and dansyl-4-n-NP, 26.9 min. Fig. 2 shows also the sufficient separation of all three dansylated xenoestrogens using the HPLC conditions described in Section 2.5.

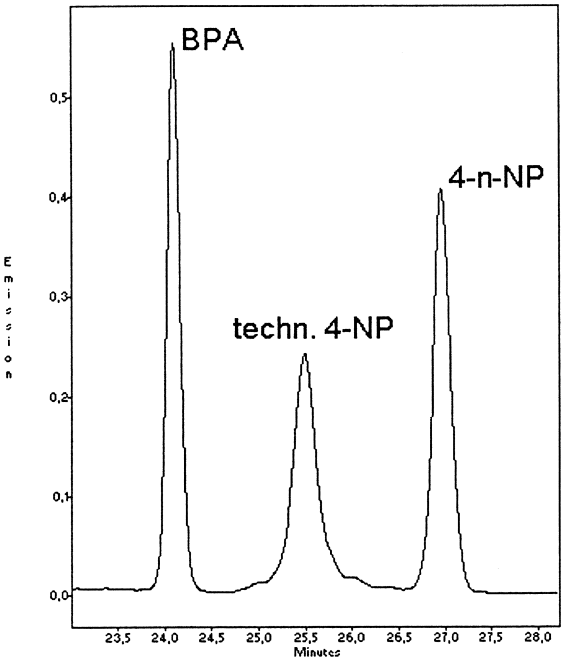

Fig. 2. HPLC chromatogram of dansylated bisphenol A, dansylated technical 4-nonylphenol and dansylated 4-*n*-nonylphenol.

Due to the fact that the phenols react quantitatively with dansyl chloride (see Section 2.3), the measured fluorescence is proportional to the amount of phenols in a sample. Consequently, all amounts given in this work are theoretical amounts on-column of underivatised phenols. The detection limits are 0.1 ng for BPA (S/N 5.1) and technical 4-NP (S/N 3.6) each and 0.5 ng for 4-n-NP (S/N 4.5). Quantification is possible within a range from 0.5 to 10 ng of substance.

4-NP and BPA can also be detected by their natural fluorescence at $\lambda_{ex}=277$ and $\lambda_{em}=300$ nm, giving the same detection limits as we found [10]. In highly encumbered samples like extracts of sewage sludge we found complex HPLC chromatograms using these wavelengths. Therefore it is not possible to apply these conditions on sewage sludge extracts.

3.2. Linearity and reproducibility of the method

For calibration, standard solutions (see Section 2.2) were derivatised as described (see Section 2.3). Standardisation by peak area was used for the quantitative determination of BPA, technical 4-NP and 4-n-NP (as internal standard). For each compound three individual calibrations between the amount of substance (as described in Section 3.1) and the peak area were performed.

Table 1 shows the corresponding equations and correlation coefficients. A linear correlation between absolute amount of substance and peak area was found in a range from 0.5 ng to 10 ng absolute. The calibration measurements for BPA and 4-n-NP were performed without using an internal standard which gave correlation coefficients >0.989 (see Table 1).

Technical 4-NP was determined using 4-n-NP as internal standard. Thus, a correlation coefficient above 0.999 was obtained (see Table 1).

3.3. Application of the method to sewage sludge

In order to test the applicability of the method for environmental samples, we performed quantivative measurements of BPA and 4-n-NP (as internal standard) from sewage sludge as described in Section 2.5. Technical 4-NP was not used in this experiments because we expected that the sludge would be contaminated with 4-NP.

Table 1
Calibration curves of bisphenol A, 4-*n*-nonylphenol and technical nonylphenol

Chemical	Experiment	Equation	Datapoints	Correlation coefficient
BPA	1	$y=84\,718x-11\,712$	4	0.9981
	2	$y=80\,138x+5057$	3	0.9995
	3	$y=80\,366x+16\,009$	4	0.9960
4-n-NP	1	$y=84\,505x+1242$	4	0.9919
	2	$y=73\,631x-3845$	4	0.9955
	3	$y=82\,924x+11\,670$	5	0.9894
Technical 4-NP	1	$y=68\,644x+53\,159$	5	0.9995
	2	$y=67\,655x+9146$	4	0.9998
	3	$y=65\,794x+5186$	5	1.0000

x=theoretical amount-on-column of underivatised substance [ng] (see Section 3.1), y=peak area.
For each substance, three calibrations independent from each other were performed as described in Section 2.2.

It should be mentioned that a complete extraction of both BPA and NP from sewage sludge is considerably difficult, and we did not aim to optimise extraction during the experiments described below (for recovery see Section 2.6). The bad recovery of BPA can be explained by its low solubility in cyclohexane.

The chromatograms of the extracts of sewage sludge are compared to those of BPA and 4-n-NP and to those of unspiked sludge (Fig. 3). The unspiked control did not show any peak of 4-n-NP and BPA. In the analysis of the spiked sludge the dansylated 4-n-NP and BPA could be separated well from dansylated natural components of the sewage sludge. In the extracts of both spiked and unspiked sludge we detected technical 4-NP as expected.

For solutions containing sewage sludge it was confirmed that the selective detection of fluorescing

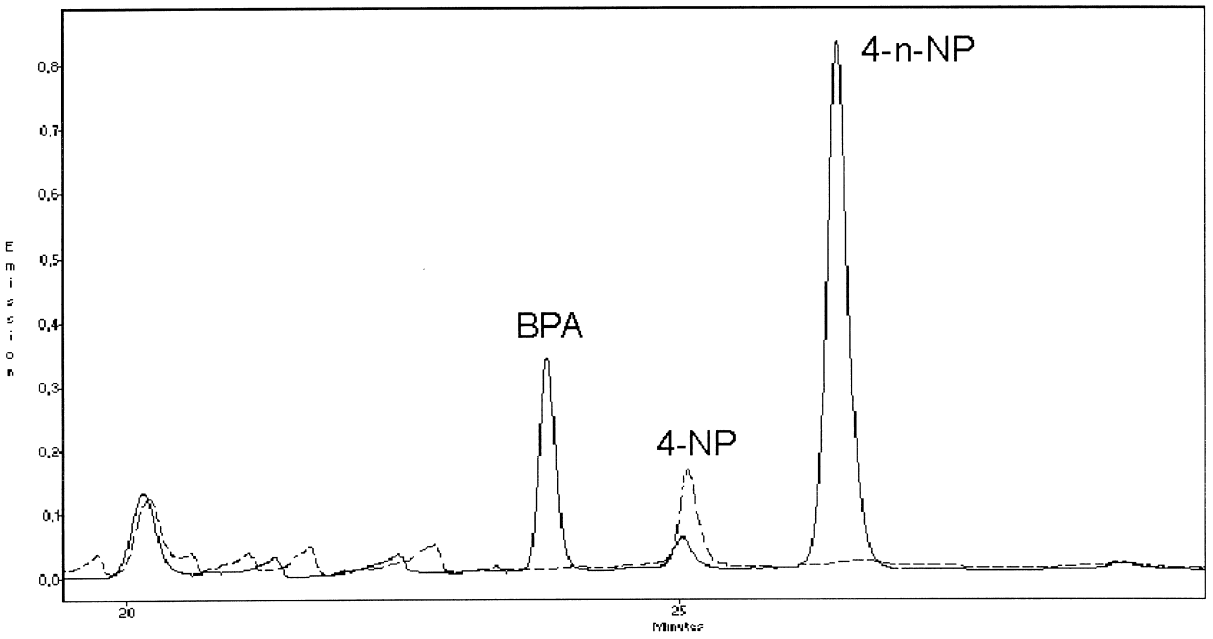

Fig. 3. Comparison of HPLC chromatograms of dansylated extracts from spiked (continuous curve; 10 μg of BPA and 4-n-NP each) and unspiked (interrupted curve) sewage sludge.

derivatives diminishes the complexity of the chromatograms and therefore reduces the requirement for sample clean-up.

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