Development and Validation of a GC/MS-Method for Determination of Phenolic Xenoestrogens in Aquatic Samples

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Introduction

In the last few years, up to about 40 non-steroidal anthropogenic substances have been identified to mimic the effects of the natural estrogen 17ß-estradiol [1-4]. Many of these xenoestrogens are phenolic compounds: *In vitro* and partly *in vivo* studies have demonstrated an estrogenic activity for 4-nonylphenol and 4-octylphenol, biodegradation products of nonionic surfactants, Bisphenol A, the monomer used in the manufacture of epoxide and polycarbonate resins, 3-t-butyl-4-hydroxyanisole, a synthetic food antioxidant, and for the multiply used industrial chemical 4-hydroxybiphenyl (e.g. production of azo dyes) [1-8]. Recently, applying a modified proliferation test with MCF-7 breast cancer cells (E-Screen assay) we could identify a weak estrogenic potency for the disinfectant 4-chloro-3-methylphenol, the herbicide educt 4-chloro-2-methylphenol, the fungicide 2-hydroxybiphenyl, and for 2-t-butyl-4-methylphenol [4, 9].

Because of their widespread application, these phenolic xenoestrogens are expected to end up primarily in the aquatic environment via sewage. Recently, it was demonstrated in the UK [10, 11] and the USA [12] that male fish held in treated sewage effluents or in rivers below sewage plants showed a pronounced increase of plasma vitellogenin levels. Therefore sewage plant effluents appear to be the major route for the release of estrogenic substances into the aquatic environment. Several investigations proved the occurrence and persistence of 4-nonylphenol [13-15] and Bisphenol A [16] in sewage plants and surface water. These findings underpin the general need for analytical monitoring data of phenolic chemicals in sewage and surface water.

We developed a GC/MS method for the simultaneous quantitative determination of a variety of structurally different phenolic xenoestrogens in surface water and sewage. Special emphasis was put on low detection limits and on a wide range of determination. Solid phase extraction (SPE) was applied as a quick extraction method which requires very little solvent. Two different solid phases were compared with regard to recoveries and handling.

Material and Methods

Chemicals: A stock solution of all reference standards in a concentration of 100 µg/ml was prepared in methanol: 4-t-octylphenol >90% purity, techn. 4-nonylphenol with ~85% content of p-isomers, Bisphenol A ~97%, 3-t-butyl-4-hydroxyanisole >98%, 4-hydroxybiphenyl >98%, 2-hydroxybiphenyl >98% (all obtained from Fluka, Buchs, Switzerland), 4-chloro-3-methylphenol 99%, 4-chloro-2-methyl-phenol 97%, and 2-t-butyl-4-methylphenol 99% (all purchased from

Aldrich, Steinheim, Germany). The corresponding working standard solutions were obtained by dilution of the stock solution with methanol. Biphenyl >99% (Merck, Darmstadt, Germany),used as internal standard, was dissolved separately in methanol to a concentration of 103 µg/ml.

Extraction: Two different solid phases, 1 g of the reversed phase $C18_{nec}$ (6 ml reservoir) and 200 mg of the polystyrene copolymer resin ENV+ (6 ml), both from ICT (Bad Homburg, Germany), were applied for solid phase extraction (SPE). Prior to the extraction step each SPE column was conditioned with 6 ml acetone, 10 ml methanol and 6 ml deionized water (pH 2).
For determination of the recoveries, 1 L of deionized water was spiked with 1 ml of the 1:1000 working standard solution (100 ng per compound). Then 5 ml methanol, H_2SO_4 for pH adjustment to 2 - 3, and different amounts of NaCl (0, 5, and 10 g) were added. Extraction of the water sample was performed at a flow rate of 10-15 ml/min. After washing with 6 ml deionized water (pH 2) and drying of the column, the phenolic compounds were eluted with 2 x 2.5 ml acetone and the solvent was evaporated to 0.5 ml with a gentle stream of nitrogen.

GC/MS analysis: An aliquot of 50 µl of the extract or standard solution respectively, was methylated with 50 µl of phenyltrimethylammoniumhydoxide (0.1 M solution in methanol purchased from Fluka, Buchs, Switzerland) at room temperature, then 10 µl of the biphenyl solution were added as internal standard.
HRGC/LRMS analysis of the phenolic xenoestrogens was carried out using a HP 5890 Series II gas chromatograph directly coupled to a HP 5972 A mass selective detector. Gaschromatographic separation was performed on a 15 m DB-XLB fused silica capillary column with 0.25 mm inner diameter and 0.25 µm film thickness (J&W Scientific Products, Köln, Germany). 1 µl of sample was injected by a HP autosampler, with the injection port at 240 °C in the splitless mode. The temperature of the GC/MS transfer line was 290 °C; the oven program was as follows: 80 °C for 1 min, 7 °C min^{-1} to 180 °C, 12 °C min^{-1} to 240 °C, 20 °C min^{-1} to 300 °C, 300 °C for 3 min. The carrier was gas helium with a flow rate of 1.16 ml/min.

Quantification: The quantification of the phenolic compounds was carried out by comparison of peak heights of the most intensive ion of each compound with that of the internal standard. Before each sequence of samples, the response factors were calculated separately from the analysis of the standard dilutions 1:10 until 1:5000 representing a concentration range of a factor 500 for quantitative determination.

Results and Discussion

Table 1 shows the m/z values applied for quantification and confirmation of nine phenolic xenoestrogens. For the internal standard biphenyl, m/z 154 was used for quantification and m/z 153 for confirmation. The limits of detection and determination are expressed as absolute amounts. The limits of detection represent a signal to noise ratio of 3:1. For assessing the limits of determination the standard dilutions 1:10 until 1:10000 were analysed and the individual response factors calculated. The linear range of determination was established for each compound separately. The lower limit of determination is shown in table 1. The results of the recoveries of nine phenolic xenoestrogens after extraction of 1 L deionized water on the ENV+ solid phase are summarized in figure 1. With the exception of 3-t-butyl-4-hydroxyanisole the recoveries of all other compounds were above 60%. Addition of NaCl to the sample elevated the recoveries up to 70% to 100%. Especially the recoveries of 4-hydroxybiphenyl, 2-hydroxybiphenyl and 2-t-butyl-4-methylphenol showed a clear improvement.

Table 1: M/z values used for quantification and confirmation and limits of detection and lower limits of determination for the GC/MS analysis of phenolic xenoestrogens in water.

	m/z quantification	m/z confirmation	limit of detection [pg absolute]	lower limit of determination [pg absolute]
4-t-octylphenol (4tOP)	149	121	<4.5	9.1
techn. 4-nonylphenol (4NP)*	149	121	5.1	25.5
Bisphenol A (BPA)	241	256	<4.7	46.8
3-t-butyl-4-OH-anisole (3tB4OHA)	179	151	4.7	23.4
2-t-butyl-4-methylphenol (2tB4MP)	163	135	4.7	4.7
4-OH-biphenyl (4OHBiP)	169	184	<4.6	23.2
2-OH-biphenyl (2OHBiP)	169	184	<4.7	23.6
4-Cl-3-methylphenol (4Cl3MP)	156	158	<5.0	10.0
4-Cl-2-methylphenol (4Cl2MP)	156	158	<5.2	10.4

* two major peaks

In the literature [17-19] the 'salting-out' effect is described for different phenolic substances. Therefore, the check and possible adjustment of the salt content of real water samples prior to extraction is necessary to achieve and ensure high recoveries.

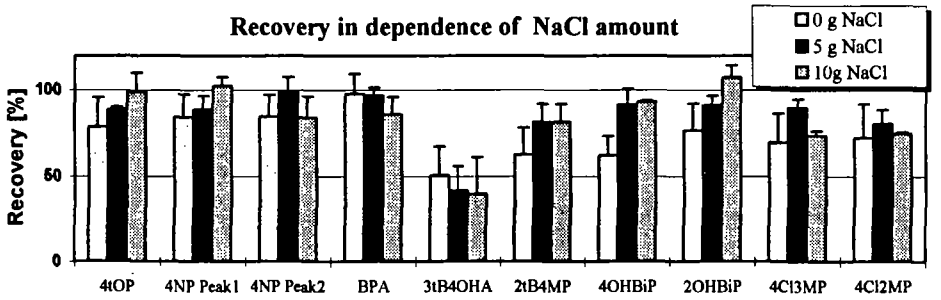

Figure 1: Recoveries of phenolic xenoestrogens after extraction of 1 l water on 200 mg of the polystyrene copolymer ENV+. Columns represent means (± SD) of three independent extractions.

The addition of H_2SO_4 and methanol to the water sample before extraction is necessary both to suppress the ionization of phenols and to condition the solid phase.

Comparing the solid phases ENV+ and $C18_{nec}$, two different aspects, recoveries and handling, were decisive for their preferred application. The recoveries of the phenolic xenoestrogens using 1 g of the $C18_{nec}$ reversed phase after addition of 5 g NaCl to the water samples were above 70% and thus similar to those achieved with the ENV+ copolymer. As on the ENV+ phase, only 3-t-butyl-4-methylanisole showed a low recovery on the $C18_{nec}$ phase. Using the $C18_{nec}$ phase extraction required more time and higher vacuum, especially when the sample contained larger amounts of suspended matter (river water). Thus, the ENV+ phase is more suitable for routine application than the C18 phase.

We analyzed real sewage samples to investigate (1) the danger of clogging of the solid phase by suspended matter and (2) the possible need of a clean up step. The application of silanized glass wool on the top of the column prevented the blockage of the solid phase by suspended particles. Figure 2 shows the total ion chromatogram of a raw sewage sample from a major municipal sewage plant in South Germany after extraction on 200 mg ENV+ solid phase. Quantitative determination of all phenolic xenoestrogens was possible without clean up.

Thus, we have established a reliable analytical method suitable for quantitative determination of various phenolic xenoestrogens in waste water which is already applied for input/output analysis of these chemicals in sewage plants [20].

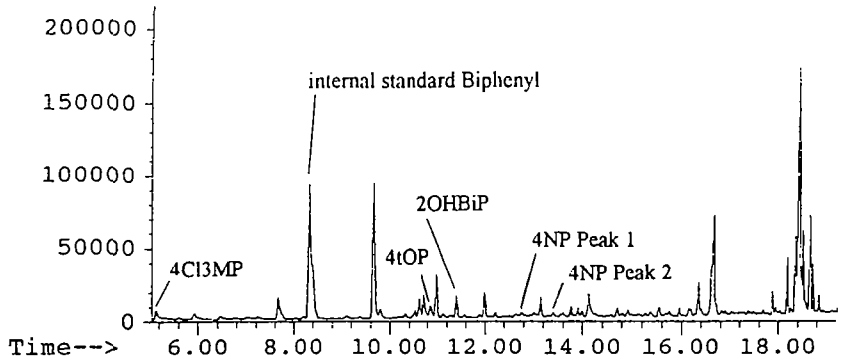

Figure 2: TIC of the HRGC/LRMS analysis (SIM mode) of a raw sewage sample from a major municipal sewage plant in South Germany after solid phase extraction on a polystyrene copolymer and methylation.

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