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Development and validation of a GC/MS method for determination of phenolic xenoestrogens in aquatic samples

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Abstract

A simple and sensitive GC/MS method for the quantitative determination of the estrogenic phenolic compounds 4-nonylphenol, 4-*t*-octylphenol, bisphenol A, 3-*t*-butyl-4-hydroxyanisole, 2-*t*-butyl-4-methylphenol, 4-hydroxybiphenyl, 2-hydroxybiphenyl, 4-chloro-3-methylphenol, and 4-chloro-2-methylphenol in aquatic samples was developed. The method for assessing their occurrence in sewage, surface and drinking waters consists of solid phase extraction (SPE) using a polystyrene copolymer phase. After methylation of the extract HRGC/LRMS analysis was possible without any clean up, even in raw sewage samples. Limits of detection and determination were between <0.01 and 0.05 ng/l and 0.01 and 0.05 ng/l, respectively. Recoveries were above 70% with exception of 3-*t*-butyl-4-hydroxyanisole.

1. 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 17β-estradiol (Soto et al., 1995; Jobling et al., 1995; Klotz et al., 1996; Körner et al., 1998). 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 non-ionic 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 widely used industrial chemical 4-hydroxybiphenyl (e.g. production of azo dyes) (Dodds and Lawson, 1936; Bitman and Cecil, 1970; Jobling and Sumpter, 1993; Krishnan et al., 1993; Jobling et al., 1995; Soto et al., 1995; Klotz et al., 1996; Körner et al., 1998). Using a modified proliferation test with MCF-7 breast cancer cells (E-Screen assay), we were able to identify a weak estrogenic activity 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 (Körner et al., 1997, 1998).

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 (Harries et al., 1996; Folmar et al., 1996) and the USA (Purdom et al., 1994) that male fish held in treated sewage effluents or in rivers below sewage plants showed a pronounced increase of plasma vitellogenin levels. Thus, 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 (Stephanou and Giger, 1982; Wahlberg et al., 1990; Field and Reed, 1996) and bisphenol A (del Olmo et al., 1997) in sewage plants and surface water. These findings demonstrate the general need for analytical monitoring data of phenolic chemicals in sewage and surface water.

Therefore, 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 placed 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.

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Two different solid phase materials were compared with regard to recoveries and handling and elution with different solvents was tested to maximize recoveries.

2. Materials and methods

2.1. 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 for quantification, was dissolved separately in methanol to a concentration of 103 μg/ml. All solvents (Promochem, Wesel, Germany) were of nanograde purity. NaCl (analytical grade) was treated at 400°C for 4 h.

2.2. Extraction

Two different solid phase materials, 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 SPE. Prior to the extraction step each SPE column was conditioned by rinsing successively 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 under nitrogen, the phenolic compounds were eluted with 2×2.5 ml acetone and the solvent was evaporated to 0.5 ml with a gentle stream of nitrogen. Elutions were also performed with 2×2.5 ml ethylacetate and the recoveries were compared with those of the elution with acetone. The extracts were stored in glass vials at 4°C until analyzed by GC/MS.

2.3. Sample preparation

Water sample were collected in brown glass bottles and stored at 4°C. First they were adjusted to room temperature, then, after pH adjustment to pH 2–3 and addition of 5 ml methanol, small portions of NaCl were added to the water sample until a conductivity equal to that of a pure aqueous 0.5% NaCl solution was achieved. Silanized glass wool (Mallinckrodt Baker, Griesheim, Germany) was added to the top of the solid phase column to prevent clogging by suspended particles during extraction.

2.4. 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, 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. Gaschromatoghraphic 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). The mass spectrometer was operated in the selected ion monitoring (SIM) mode to detect the methylether of the phenolic compounds. One 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 temperature program was as follows: 80°C for 1 min, 7°C/min to 180°C, 12°C/min to 240°C, 20°C/min to 300°C, 300°C for 3 min. The carrier was gas helium (purity 4.6, Messer, Griesheim, Germany) with a flow rate of 1.16 ml/min.

2.5. 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 1:10 to 1:5000 dilutions of the stock solution representing a concentration range of a factor of 500 for quantitative determination.

3. Results

3.1. Determination and quantification

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 in absolute

amounts. The limits of detection represent a signal to noise ratio of 3:1. For assessing the limits of determination, the 1:10 to 1:10 000 (5 ng–5 pg absolute) dilutions of the stock solution were analyzed and the individual response factors calculated. The linear range of determination was established for each compound separately. The linearity of the range of determination is presented in Fig. 1. The lower limits of determination are also shown in Table 1.

3.2. Limits of detection and determination

To check whether the instrumental limits of detection and determination corresponded to the limits of the analytical method, 1 l deionized water was spiked with the 1:5000 (20 ng/l of each compound) and 1:10 000 (10 ng/l) dilutions of the stock solution and the recoveries were determined.

The recoveries at 20 ng/l found in this experiment ($n = 3$) ranged between 50% and 120% for 4-*t*-octylphenol, techn. 4-nonylphenol, 4-hydroxybiphenyl, 2-hydroxybiphenyl, 4-chloro-3-methylphenol, 4-chloro-2-methylphenol and 2-*t*-butyl-4-methylphenol. Bisphenol A showed a recovery higher than 140%, indicating that the limit of determination lies above 20 ng/l. 3-*t*-Butyl-4-hydroxyanisole was no longer detectable at a concentration of 20 ng/l. Table 2 shows the resulting methodical limits of detection and determination.

3.3. Technical 4-nonylphenol

Technical 4-nonylphenol consists of a mixture of *p*-isomers, consequently a complex pattern of peaks will be obtained in the mass fragmentograms by GC/MS analysis. The identification of the different *p*-isomers is not yet possible because the individual pure standard compounds are currently not available (Thiele et al., 1997). For the purpose of easy and reliable quantification we decided to determine the sum of 4-NP isomers by comparison of the peak heights of two major peaks in the technical mixture. Following the numerical order of 16 separated peaks of Thiele et al. (1997) we quantified peak 3 and peak 15 of $m/z = 149$, equivalent to $m/z = 135$ of the underivatized 4-NP. The evaluation of the peak

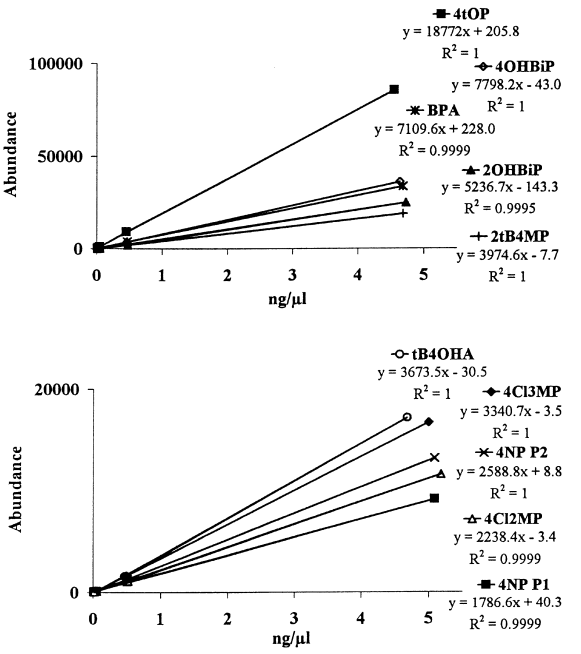

Fig. 1. Linearity of the determination range of phenolic xenoestrogens between 5 ng and 5 pg (absolute).

Table 1
m/z values used for quantification and confirmation, limits of detection and lower limits of determination for the GC/MS analysis of phenolic xenoestrogens

	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)[a]	149	121	5.1	10.2
Bisphenol A (BPA)	241	256	<4.7	20.4
3-*t*-butyl-4-OH-anisole (3tB4OHA)	179	151	4.7	9.4
2-*t*-butyl-4-methylphenol (2tB4MP)	163	135	4.7	9.4
4-OH-biphenyl (4OHBiP)	169	184	<4.6	9.3
2-OH-biphenyl (2OHBiP)	169	184	<4.7	9.5
4-Cl-3-methylphenol (4Cl3MP)	156	158	<5.0	5.0
4-Cl-2-methylphenol (4Cl2MP)	156	158	<5.2	5.2

[a] Quantification of total 4NP via average of peaks 3 and 15.

Table 2
Limits of detection and lower limits of determination for the analysis of phenolic xenoestrogens in 1 l demineralized water[a]

	Limit of detection (ng/l)	Lower limit of determination (ng/l)
4-*t*-octylphenol (4tOP)	<0.01	0.02
techn. 4-nonylphenol (4NP)[b]	0.01	0.02
Bisphenol A (BPA)	<0.01	0.05
3-*t*-butyl-4-OH-anisole (3tB4OHA)	0.05	0.05
2-*t*-butyl-4-methylphenol (2tB4MP)	0.01	0.02
4-OH-biphenyl (4OHBiP)	0.01	0.02
2-OH-biphenyl (2OHBiP)	0.01	0.02
4-Cl-3-methylphenol (4Cl3MP)	<0.01	0.01
4-Cl-2-methylphenol (4Cl2MP)	<0.01	0.01

[a] Results were determined from three independent extractions.
[b] Quantification of total 4NP via average of peak 3 and 15.

heights of single selected peaks rather than the sum of all isomers reduces the risk of falsification by coeluting substances. Fig. 2 shows the $m/z = 149$ traces of a raw sewage sample in comparison with that of the standard of techn. 4-nonylphenol. By integration of all isomers of techn. 4-nonylphenol the marked unknown peak would be co-quantified, resulting in an erroneously high apparent concentration of over 140%.

3.4. Recoveries

The results of the recoveries of nine phenolic xenoestrogens after extraction of 1 l deionized water on the ENV+ solid phase and elution with 2×2.5 ml acetone are summarized in Fig. 3. With the exception of 3-*t*-butyl-4-hydroxyanisole, the recoveries of all other compounds were above 60%. Addition of 5 g NaCl to the sample generally elevated the recoveries to 80–100%. Especially the recoveries of 4-hydroxybiphenyl and 2-*t*-butyl-4-methylphenol showed a clear improvement. Increasing the amount of NaCl to 10 g per l reduced the recoveries, particular those of bisphenol A which were even under the recoveries without addition of NaCl. These results indicated that the optimum salt content lies at 5 g NaCl per l and therefore the checking and, if necessary, adjustment of the salt content of real water samples prior to extraction is necessary to achieve and ensure high recoveries. In the literature Chladek and Marano (1984), Heberer and Stan (1997) and Bao et al. (1996), the 'salting-out' effect has been described for different phenolic substances.

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

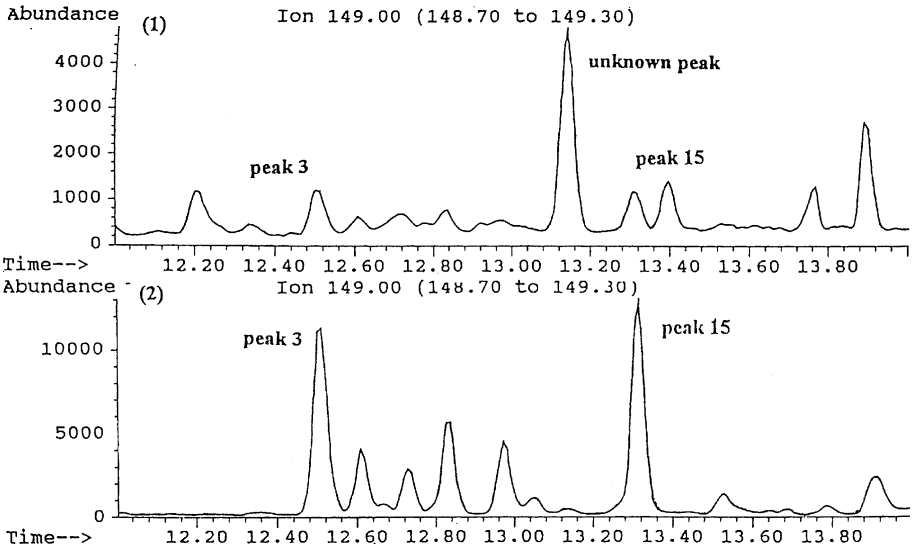

Fig. 2. $m/z = 149$ traces of a raw sewage sample in comparison with that of the standard of techn. 4NP.

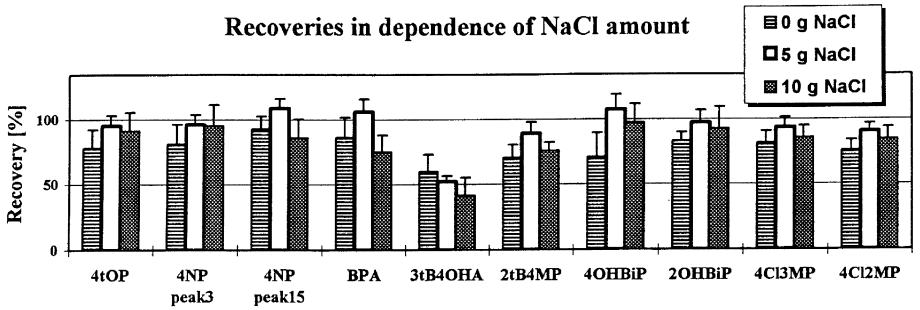

Fig. 3. Recoveries of phenolic xenoestrogens after extraction of 1 l water on 200 mg of the polystyrene copolymer ENV+. Columns represent means (±SD) of five independent extractions.

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 (Table 3). As on the ENV+ phase, only 3-*t*-butyl-4-methylanisole showed a low recovery on the $C18_{nec}$ phase. Use of 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. When 2×2.5 ml ethylacetate was used for elution with ENV+ as solid phase, comparable recoveries were achieved with the exception of 3-*t*-butyl-4-methylanisole (see Table 3). However, when eluting with ethylacetate, the column must be completely dry before elution because ethylacetate and water are not miscible and a two-phase mixture would be obtained. To clarify whether adsorption on the solid phase is the reason for the low recovery of 3-*t*-butyl-4-hydroxyanisole, deionized water was spiked with higher amounts of 3-*t*-butyl-4-hydroxyanisole (500 and 1000 ng/l). The recoveries for 3-*t*-butyl-4-hydroxyanisole did not increase at both concentrations, thus eliminating adsorption as the source of the low recovery.

3.5. Internal standard

Biphenyl was selected as internal standard for quantification because of its similar gaschromatographic retention and formation of an intense molecule ion peak after electron impact ionization. The absence of a hydroxy group prevents the molecule to be methylated, consequently no chemical reaction is expected to occur during derivatization. As biphenyl is used as a fungicide and insecticide, its occurrence in the aquatic environment could be possible. Therefore, when real water samples are analyzed, the absence of biphenyl in the sample must be proved. Alternatively we use D_{10}-biphenyl as internal standard (*m*/*z* 164, 163).

3.6. Sewage samples

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 reproducibility was also investigated by multiple analysis of a 24 h sewage sample. The use of silanized glass wool on the top of the column prevented blockage of the solid phase by suspended particles. Fig. 4 shows the total

Table 3
Comparison of recoveries of phenolic xenoestrogens using a $C18_{nec}$ or ENV+ solid phase[a]

Recovery (%), addition of 5 g NaCl	$C18_{nec}$, elution with acetone	ENV+, elution with acetone	ENV+, elution with ethylacetate
4-*t*-octylphenol (4tOP)	97 ± 12	95 ± 8	99 ± 8
techn. 4-nonylphenol (4NP) peak 3	114 ± 23	96 ± 8	110 ± 6
techn. 4-nonylphenol (4NP) peak 15	111 ± 24	109 ± 8	117 ± 8
Bisphenol A (BPA)	105 ± 18	106 ± 10	101 ± 7
3-*t*-butyl-4-OH-anisole (3tB4OHA)	25 ± 12	52 ± 5	7 ± 2
2-*t*-butyl-4-methylphenol (2tB4MP)	73 ± 9	89 ± 9	66 ± 13
4-OH-biphenyl (4OHBiP)	102 ± 15	107 ± 12	102 ± 10
2-OH-biphenyl (2OHBiP)	102 ± 15	97 ± 9	96 ± 9
4-Cl-3-methylphenol (4Cl3MP)	90 ± 6	93 ± 8	90 ± 6
4-Cl-2-methylphenol (4Cl2MP)	89 ± 11	90 ± 6	79 ± 6

[a] The elution was performed once with acetone and once with ethylacetate. Values represent means ±SD of five independent experiments.

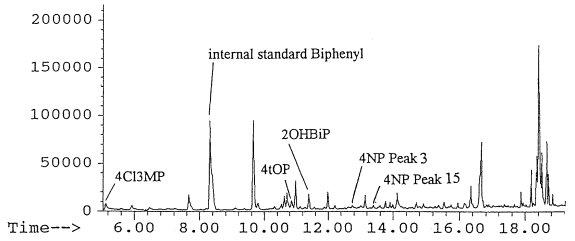

Fig. 4. Total ion chromatogram of the HRGC/LRMS analysis (SIM mode) of a raw sewage sample from a major municipal sewage plant in South Germany after solid phase extraction on a polystyrene copolymer resin and methylation.

ion chromatogram of a raw sewage sample from a major municipal sewage plant in South Germany after extraction on 200 mg ENV+ solid phase and elution with acetone. Quantitative determination of all phenolic xenoestrogens was possible without clean up.

Three independent extractions and analysis of 1 l aliquots of the same 24 h effluent sample of a municipal sewage plant in South Germany demonstrated the reproducibility of the developed method. The concentrations of the identified phenolic xenoestrogens 4-*t*-octylphenol, 4-nonylphenol and bisphenol A were between 0.2 and 0.4 μg/l (compare with Fig. 5), the standard deviations of the triple analysis ranged between 31% and 4%. 4-Nonylphenol has already been investigated as to its enrichment and persistence in sediments and sewage sludge (Wahlberg et al., 1990; Lee and Peart, 1995). With a $\log K_{ow}$ (octanol–water partition coefficient) of 4.48, 4-nonylphenol is also expected to be adsorbed on the particulate phase of a water sample. Since the amount of suspended particulate matter differed in each single aliquot of the 24 h effluent sample, varying concentrations of 4-nonylphenol were found as a result of co-extraction of the particulate phase, which accumulated on the silanized glass wool at the top of the SPE column. This aspect may explain the higher deviation of the 4-nonylphenol concentrations. Fig. 5 also shows that the determination of the sum of 4-nonylphenol isomers is possible by quantification as described above, both quantified peaks (peaks 3 and 15) led to the same mean concentration for the sum of techn. 4-nonylphenol.

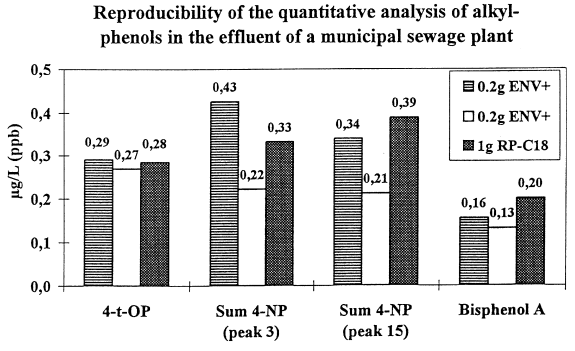

Fig. 5. Three independent analyses of a 1 l aliquots of a 24 h effluent sample from a municipal sewage plant using 0.2 g ENV+ and 1 g RP-$C18_{nec}$ as solid phases. Elution was performed with acetone.

4. Conclusion

We were able to establish a sensitive and reliable analytical method suitable for quantitative determination of nine structurally different phenolic xenoestrogens in water, applicable also for wastewater. After SPE of 1 l sample and methylation, GC/MS analysis was possible without any clean up. The limits of determination were between 10 and 50 ng/l. The method has already successfully been applied for the monitoring of these phenolic chemicals in various compartments of the aquatic environment, e.g. river water, and for input/output analysis in municipal sewage plants (Körner et al., 2000).

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