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Determination of endocrine disruptors in water after derivatization with *N*-methyl-*N*-(*tert*.-butyldimethyltrifluoroacetamide) using gas chromatography with mass spectrometric detection

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Abstract

The combined gas chromatographic determination of a number of hydroxyl-group containing endocrine disruptors, including 4-octylphenol, 4-nonylphenol, 2,4-dichlorophenol, pentachlorophenol, 4-*tert*.-butylbenzoic acid, bisphenol-A, 17β-estradiol and 17α-ethynylestradiol, was investigated. Derivatization, required for sensitive determination of these compounds, was carried out using *N*-methyl-*N*-(*tert*.-butyldimethyltrifluoroacetamide). A number of parameters affecting the derivatization reaction, like temperature, time, matrix, solvent, and amount of reagent were studied in detail. Quantitative yields were obtained for real-life extracts after optimization, but the hormones were only mono-substituted. Both solid-phase extraction (SPE) and liquid–liquid extraction were studied as extraction methods, with emphasis on SPE material and effect of pH. Recoveries and RSD for analysis of surface water samples were 58–106 and 6–16% ($n=4$), respectively, when using SPE, and 109–117 and 6–14% ($n=6$) when using liquid–liquid extraction. The method developed allows routine analysis of surface water for traces of endocrine disruptors. The limits of detection of were 4–6 ng/l but higher for the hormones.

Keywords: Water analysis; Derivatization, GC; Environmental analysis; Endocrine disruptors; Carboxylic acids; Phenols; Hormones; Methylbutyldimethyltrifluoroacetamide

1. Introduction

In the past few years there has been a growing concern on possible harmful consequences of exposure to xenobiotic compounds that are capable of modulating or disrupting the endocrine system. This concern for endocrine disrupting chemicals is directed at both wildlife and humans [1,2,12–14]. Effects attributed to endocrine disruptors include the development of testicular and prostate cancer and reduced sperm production in humans, and demasculation, feminization, alteration of immune functions and decreased fertility in birds, fish and mammals.

A wide variety of chemicals have been identified as endocrine disruptors or are suspected to be able to affect the endocrine system. In Table 1 [7] most frequently cited compounds are given with their application. As relationships between the chemicals and the observed effects in many cases are still hypothetical, additional scientific research is required into the nature and severity of the reported phenomena, the extent to which people and wildlife are exposed and the association between the two of these.

In risk assessment of endocrine disruptors, an

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Table 1
Classes of suspected endocrine disruptors

Class	Use	Availability analytical methods
Pesticides	Agriculture, domestic use, (e.g. DDT, vinclozolin, tributyltin, pentachlorophenol)	+
Natural/synthetic hormones	−/Contraceptives, growth promotor agents	−
Alkylphenol polyethoxylates	Detergents, personal care	±
→alkyl phenols	Degradation product, anti-oxidants	−
PCBs/dioxines	Heat transfer fluids/−	+
Phtalate esters	Plasticizers	+
Phyto estrogens	Naturally occurring in plants	−
Other: bisphenol A	Polymer production	−
4-*tert.*-butylbenzoic acid	Plastics, paint	−
2,4-chlorophenol	Chemical industry	+

important aspect is knowledge about their presence and fate in the environment. For many pesticides, polychlorinated biphenols (PCBs) and dioxines methods for chemical analysis are available and applied for monitoring of these compounds in the environment for many years. To a lesser extent, this is also true for chlorophenols [3–5], phthalic esters [6], and alkylphenol ethoxylates [8–11].

For the remaining compound (classes) from Table 1, i.e. the alkyl phenols, the hormones, and miscellaneous substances like bisphenol-A and 4-*tert.*-butyl benzoic acid, only few methods for determination in environmental water have appeared in literature so far.

The determination of alkylphenols (emphasis is on pentyl- to nonylphenols) has been described using both high-performance liquid chromatography-(HPLC) and gas chromatography (GC)-based methods. Kvistad et al. [15] used solid-phase extraction (SPE) for isolation of the alkylphenols from aquaria water and was able to achieve detection limits of approximately 0.2 μg/l. Rudel et al. [16] used liquid–liquid extraction prior to HPLC–UV analysis of waste water and ground water but detection limits were limited to 2–6 μg/l. Lower detection limits were possible when using GC–mass spectrometry (MS) after silylation with bis(trimethylsilyl)trifluoroacetamide (BSTFA)+10% trimethylchlorosilane (TMCS). The derivatization was carried out by adding 25 μl of reagent to 500 μl of the dichloromethane extract. In order to obtain yields greater than 65% the mixture had to be heated to 90°C for 5 h. In reagent water good recoveries and detection limits of 5–10 ng/l were obtained, but no data were given on the derivatization yield in real water samples. Jahr [17] included nonylphenol in a method for determination of methyl-, chloro- and nitrophenols using an on-line SPE–GC–MS set up. The phenols were acetylated in the aqueous phase after pH adjustment. No recovery experiments were undertaken. For nonylphenol in tap water the limit of detection was 27 ng/l. At levels higher than 1 μg/l problems were encountered with memory effects from the on-line system. Some problems were also encountered with the stability of the acetic anhydride derivatization reagent, improper storage led to hydrolysis which resulted in considerable decrease in method sensitivity (i.e. derivatization yield).

The determination of bisphenol-A in river water has been carried out by Markham et al. [20]. An attempt was made to use HPLC with fluorescence detection but selectivity proved to be insufficient to analyze samples below the level of 3 μg/l. By GC–MS analysis of extracts obtained after liquid–liquid extraction detection limits of 1 μg/l were obtained with, using deuterated Bisphenol-A as internal standard, excellent recoveries and repeatability in both deionized and surface water. A similar method with similar detection limits was described by Olmo et al. for sea water analysis [18].

As for the alkylphenols, lower detection limits were obtained after silylation (5 ng/l, as described above, Rudel et al. [16]).

Only very few methods have been reported for determination of hormones in environmental water. 17α-Ethynylestradiol was included in the on-line SPE–GC–MS method described above [17], in tap water the acetylated derivative could be detected down to 15 ng/l. Lee et al. [19] determined 17β-estradiol and its metabolites (estrone and estriol) and 17α-ethynylestradiol in sewage effluents by GC–MS after derivatization with pentafluorpropionic acid anhydride (PFPA). For the derivatization 50 μl of reagent was added to 100 μl concentrated extract (ambient temperature, 20 min). The reaction was assumed to be quantitative as protraction of the reaction time and higher temperatures did not improve the yield. By-products formed had to be removed by washing with an aqueous K_2CO_3 solution. For effluent recoveries of 87–127% and detection limits of 5–10 ng/l were reported.

In the present study emphasis is on the combined determination of hydroxyl group containing endocrine disruptors, i.e. alkylphenols, hormones (phenolic steroids), bisphenol-A, 4-*tert*.-butylbenzoic acid and chlorophenols. The use of LC–MS–MS for this was considered initially here, but the possibility to include the current analytes in existing multi-residue methods for pesticides and PCBs, led to the choice of GC–MS as analysis technique, even though derivatization is expectedly required for obtaining low detection limits. After verification of the need for derivatization and selection of *N*-methyl-*N*-(*tert*.-butyldimethyltrifluoroacetamide) (MTBSTFA) as suitable derivatization reagent, optimization of the derivatization is described in detail for standards as well as surface water extracts and several extraction methods are compared.

2. Experimental

2.1. Chemicals

Ethyl acetate and hexane were obtained from Mallinkrodt Baker (Deventer, Netherlands), dichloromethane from Promochem (Wesel, Germany) and acetonitrile from Biosolve (Valkenswaard, Netherlands). All solvents were pesticide grade. The derivatization reagent MTBSTFA >97% was purchased from Fluka (Zwijndrecht, Netherlands).

All analytes had a purity of 98% of higher. 4-*n*-Octylphenol was obtained from Brunschwig Chemie (Amsterdam, Netherlands), 4-*n*-nonylphenol from Lancaster (Morecamle, UK), 2,4-dichlorophenol and pentachlorophenol from C.N. Schmidt (Amsterdam, Netherlands), 4-*tert*.-butylbenzoic acid, bisphenol-A, 17β-estradiol and 17α-ethynylestradiol from Aldrich (Zwijndrecht, Netherlands).

All standard solutions were prepared in ethyl acetate.

2.2. Sample preparation

Surface water was a mixture of samples taken from ditches and canals from several locations in the Netherlands and considered representative for the samples to be analyzed after implementation of the method.

2.2.1. Extraction

Water was extracted by SPE or liquid–liquid extraction. When SPE was used for surface water, a filtration step was carried out prior to the extraction. Paper (Schleicher&Schuell, Dassel, Germany) or glass fiber (type APFA, Millipore, Bedford, MA, USA) was used for this purpose. Adjustment of pH was done after filtration using sulphuric acid. For SPE, cartridges packed with a C_{18} material (1000 mg, 6 ml) or a polymer material (styrene–divinylbenzene, PS–DVB, 200 mg, 3 ml) both from Baker, Deventer, Netherlands, were used. C_{18} solid-phase extraction disks (47 mm I.D.) were obtained from 3M. Cartridges or disks were placed on a vacuum manifold, conditioned with methanol and HPLC water, after which 500 ml of water was sampled through. Excess of water remaining on the cartridge or disk was removed by suction of air for 10–15 min. The compounds retained were desorbed with two portions of 2.5 ml of ethyl acetate in case of the cartridges and three portions of 10 ml for the disks. The extracts were chemically dried with anhydrous sodium sulphate and further concentrated by evaporation under a low flow of nitrogen gas at approximately 35°C.

In case of liquid–liquid extraction, 500 ml of

water sample was extracted, after adjustment of pH, twice with 100 ml of ethyl acetate. The organic fractions were combined and concentrated to approximately 5 ml using a rotary evaporator at reduced pressure at approximately 40°C. The extract was dried using anhydrous sodium sulphate and quantitatively transferred into a calibrated tube and further concentrated by evaporation under a low flow of nitrogen gas at approximately 35°C.

2.2.2. Derivatization

In general, standard solutions and extracts were derivatized in a test tube or autosampler vial by addition of MTBSTFA and placing the mixture in a water bath at 75°C for a certain time. After derivatization, the extract was ready for injection into the GC–MS system. Details will be given in the Results and discussion section.

The final procedure for derivatization of the analytes in water extracts, as applied after optimization, was as follows: the extract was concentrated to a volume of 200 μl. Next, 200 μl of MTBSTFA was added, the tube was closed and placed in a water bath heated to 75°C for 3 h. A solution of PCB-138 was added as an internal standard for GC–MS analysis after which the volume was adjusted to 1 ml with ethyl acetate. Starting with 500 ml water samples, the concentration factor was 500.

2.3. GC–MS analysis

For GC–MS analysis a model 8000-top GC system equipped with a Best PTV injector, an AS800 autosampler and a Voyager mass spectrometer (Interscience, Breda, Netherlands) was used. The injector was equipped with a 1 mm I.D. liner without glass wool. Injection was performed in the cold splitless mode, i.e. 2 μl was injected at 70°C after which the programmed-temperature vaporizer (PTV) injector was heated to 300°C at 10°C/s and kept at that temperature for 5 min. The splitless time was 80 s. For chromatographic separation a 30 m×0.25 mm I.D. capillary column, coated with 0.25 μm 5% phenyl–dimethylsiloxane phase, was used (HP-5-MS, Boom, Meppel, Netherlands). The temperature program applied was as follows: initial temperature 50°C (1 min), at 20°C/min to 120°C (0 min), at 10°C/min to 280°C (11 min). Helium was used as carrier gas at a constant flow of 1.5 ml/min. Data acquisition was performed in full scan mode measuring from m/z 90 to 460. Data handling was performed using Masslab software.

2.4. Quantification

For quantification purposes, the response (peak area) obtained after normalization for the peak area of the internal standard, was used. Samples were injected between standards and the concentrations were calculated using the mean of the bracketing standards.

3. Results and discussion

3.1. GC–MS of parent and derivatized compounds

In principle, the analytes aimed for in this work, i.e. hydroxyl-group containing endocrine disruptors (for structures, see Fig. 1), can be determined by GC–MS without derivatization. However, several problems are usually encountered with trace level analysis in environmental samples. Losses due to adsorption in the inlet may occur. Peak tailing due to interaction of the analytes with active sites in the analytical column can be, and in fact was (see Fig. 2), observed for all compounds. The lower the amount of analyte introduced, and the older (more contaminated/active) the column, the more severe the tailing. An additional effect that complicates quantification is that this tailing is matrix dependent. Matrix can shield active sites in both the inlet and the analytical column thereby preventing losses and peak tailing [21,22]. This is illustrated in Fig. 2 for octyl and nonylphenol. The very broad peaks in the lower chromatogram improve dramatically when introduced together with matrix (surface water extract). The extent of peak sharpening, however, varies greatly with the type of water, e.g. organic matter content, which complicates quantification even when applying matrix-matched calibration. As can be seen in the two upper chromatograms in Fig. 2, sharp peaks are obtained after derivatization of the alkylphenols, even when the amount of compound introduced was 16 times less. Consequently, much lower detection limits can be obtained. The fact that

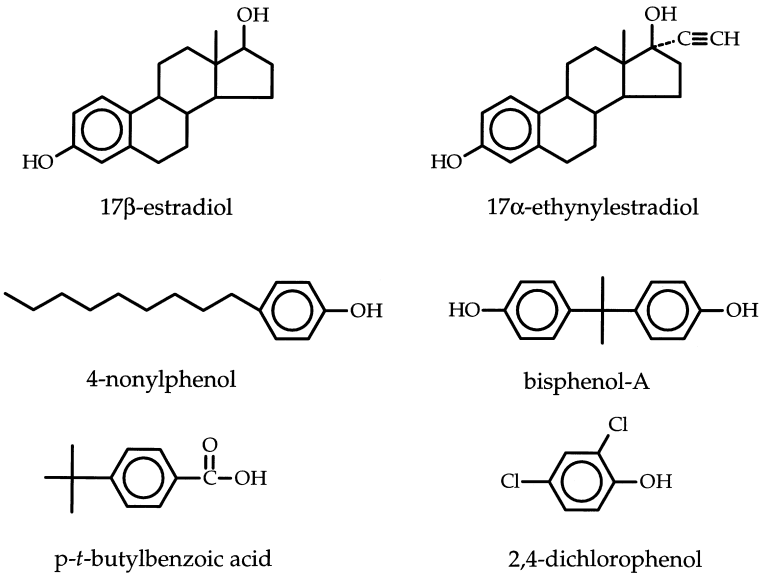

Fig. 1. Molecular structures of selected endocrine disruptors.

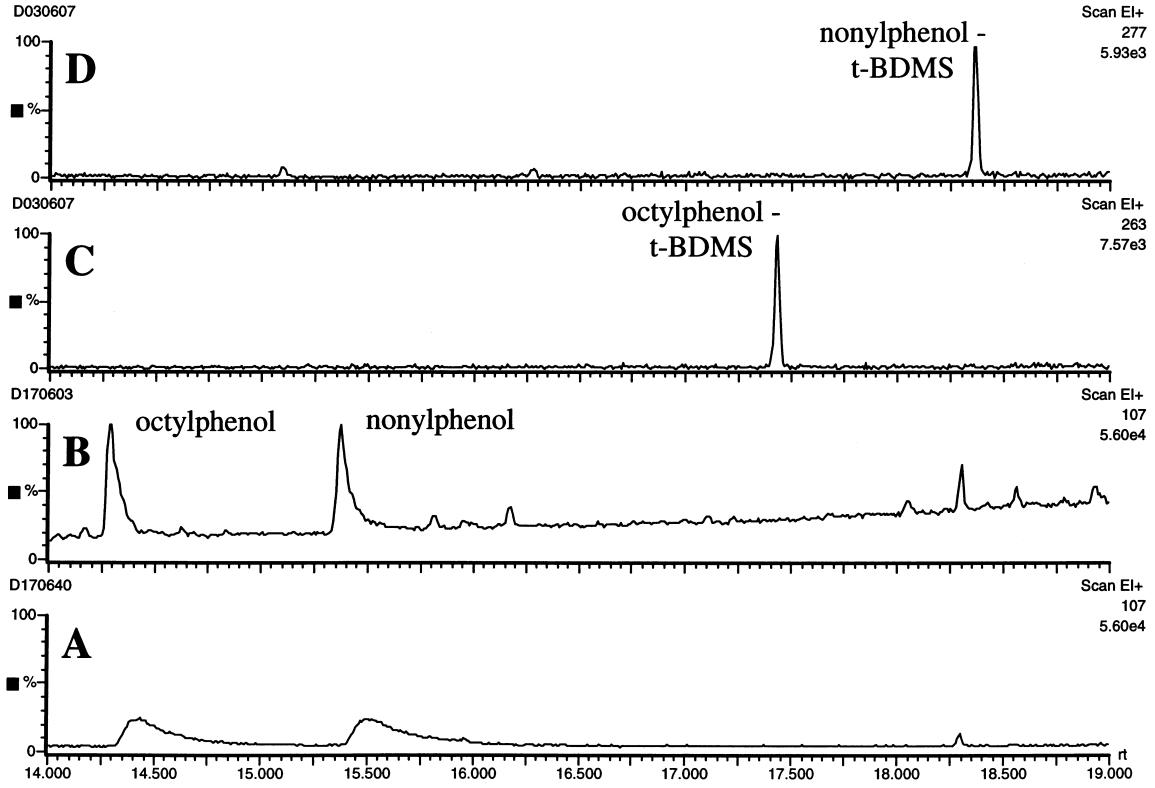

Fig. 2. Extracted ion chromatograms for 4-*n*-octylphenol and 4-*n*-nonylphenol. Standards in ethyl acetate (A) and surface water extract (B) (both 160 pg injected). *tert*.-BDMS derivatives of the octyl (C) and nonylphenol (D) (10 pg each). rt, Retention time in minutes.

ions of much higher m/z are formed for this particular derivative compared to the parent compound also contributes to this and is a third beneficial effect of derivatization of these type of compounds.

The analytes in this work are several types of phenols (alkyl, chloro, steroids) and a carboxylic acid. Some of the compounds, such as bisphenol-A and the hormones, contain two hydroxyl groups. For derivatization an agent is required that will react with all compounds. Silylation is a procedure that is suitable for, amongst others, alcohols, phenols and acids [23]. While many silyl derivatives have limited stability with regard to hydrolysis, the use of MTBSTFA results in the formation of *tert.*-butyldimethylsilyl (*tert.*-BDMS) derivatives which are much less sensitive to hydrolysis. In addition, the derivatives formed have very favorable EI mass spectra (usually a $[M\text{-}57]^+$ base peak is observed) which facilitates low detection levels [24]. The reagent has been successfully applied for trace level determination of methyl-, chloro- and nitrophenols in water [3]. For these reasons, MTBSTFA was selected as derivatization agent in this work.

For all compounds and their *tert.*-BDMS derivatives, obtained by addition of 50 μl of MTBSTFA to 1 ml of concentrated standard solutions in ethyl acetate and heating for 1 h at 75°C, retention times and mass spectra were recorded. The results are given in Table 2. Retention times increased by about 3 min for most silylated compounds compared to the non-derivatized compounds. The three major ions for the two alkylphenols were the $[M\text{-}57]^+$ (base peak), the molecular ion (abundance approximately 20%) and an ion m/z 165 corresponding to $[CH_3–C_6H_4–O–Si(CH_3)_2]^+$. The latter ion is also indicative for other *n*-alkylphenols. For the acid and both chlorophenols again $[M\text{-}57]^+$ was observed as the base peak but the molecular ion was absent. In the spectra of both chlorophenols m/z 93 and m/z 95, resulting from a dimethylsilylchloride fragments, were present with high abundance which is in agreement with earlier findings for chlorophenols [3]. For bisphenol-A both hydroxyl groups had reacted with MTBSTFA, as was evidenced by the presence of the molecular ion m/z 456. The base peak m/z 207 corresponded to $[$*tert.*-BDMS$–O–C_6H_4]^+$. Bisphenol-A was the only compound for which the typical $[M\text{-}57]^+$ ion was not observed. The two hormones were only mono-substituted after derivatization, besides the $[M\text{-}57]^+$ the molecular ion was present with an abundance of 40–60%. The expectation that only the hydroxyl group of the unsaturated ring in the molecule (3-OH) reacted (more acidic than the 17-OH group and less sterically hindered) was confirmed by the presence of m/z 163 in the mass spectra of both hormones which can be attributed to the fragment $[(CH_3)_2Si–O–C_6H_3–CH_2]^+$. Reaction at the 3-OH was further evidenced by the fact that mestranol did not react at all. Mestranol has the same structure as ethynylestradiol but has a methoxy instead of a hydroxyl group attached to the unsaturated ring. The inability to

Table 2
Retention times and mass spectrometric data for endocrine disruptors and their *tert.*-BDMS derivatives

Compound	Not derivatized		*tert.*-BDMS derivative	
	t_r (min)[a]	Diagnostic ions	t_r (min)[a]	Diagnostic ions
2,4-Dichlorophenol	7.4	162, 164, 98	12.6	219, 221, 95, 93
tert.-Butylbenzoic acid	11.1	163, 135, 91	14.8	235, 191, 161
Pentachlorophenol	13.5	266, 264, 262	18.1	323, 325, 95, 93
4-Octylphenol	14.1	107, 206	17.1	263, 165, 320
4-Nonylphenol	15.2	107, 220	18.0	277, 165, 334
Bisphenol-A	18.4	213, 228, 119	22.9	441, 207, 456
17β-Estradiol	21.5	272, 213, 160	26.1	329, 386, 330
17α-Ethynylestradiol	23.2	296, 160	27.3	353, 410, 327

[a] GC conditions: 30 m×0.25 mm I.D., 0.25 μm HP-5-MS; 50°C (1 min) – 20°C/min – 120°C (0 min) – 10°C/min – 280°C (11 min), helium 1.5 ml/min.

substitute the 17-OH of ethynylestradiol was also observed by others when using pentafluorpropionic acid anhydride (PFPA) as derivatization reagent [19].

3.2. Optimization of derivatization

There are a number of parameters that can affect the derivatization: time, temperature, solvent, matrix, amount of reagent. All these parameters were investigated. Initially this was done at the relatively high analyte concentration level of 20 μg/ml. At this level both underivatized and *tert.*-BMDS-labelled compounds could easily be monitored using GC–MS analysis.

3.2.1. Effect of reaction time

Starting from the initial conditions mentioned above (addition of 50 μl of MTBSTFA to 1 ml of a 20 μg/ml solution in ethyl acetate, 75°C), reactions were carried out in triplicate at several reaction times, i.e. 15, 30, 60 and 180 min. When reaction times of 30 min or longer were applied, no underivatized compounds were present in the chromatograms and no significant increase in the response of the derivatives was observed. There were no indications for substitution of the 17-OH of the hormones. Decreasing the reaction time to 15 min had an adverse effect on the repeatability of the response [relative standard deviations (RSDs) of approximately 20% compared to 0.5–8% at ≥30 min]. Furthermore, additional experiments with 15 min reaction times revealed that low-yield outliers occurred every now and then. It was concluded that 30 min was the minimal time required for quantitative reaction of the endocrine disruptors in the standard solutions.

3.2.2. Effect of temperature

To evaluate whether or not it was really necessary to perform the derivatization at increased temperature the reaction was also conducted at ambient temperature. The reaction times applied were 30 min, and 1 h. Experiments were conducted in 3- or 5-fold several times. Low-yield outliers were observed frequently. Apparently, the reaction is not very robust at room temperature. As satisfactory results were obtained at 75°C, no attempts were made to use higher temperatures.

3.2.3. Effect of the solvent

It is known from literature that the solvent can effect the reaction [23]. To evaluate how critical the choice of solvent was for the derivatization, four solvents commonly used in liquid–liquid and/or solid-phase extraction, were examined. Autosampler vials were filled with 950 μl of ethyl acetate, acetonitrile, dichloromethane or hexane. Next, 50 μl of standard solution in ethyl acetate was added and mixed. Then 50 μl of MTBSTFA was added, the vial was closed and placed in a water bath for 30 min or 3 h after which the extracts were analyzed by GC–MS. The results are shown in Fig. 3. The yields were compound, solvent and time dependent. Ethyl acetate proofed to be a very favorable solvent for the reaction. Results obtained for acetonitrile were comparable. Except for the more acidic ones, i.e. the benzoic acid and both chlorophenols, the yields in the less polar solvents dichloromethane and hexane were low for all compounds. Increasing the reaction time hardly improved this. For the carboxylic acid and both chlorophenols the selection of the solvent was not very critical.

3.2.4. Effect of moisture

For silylation reactions and the derivatives formed it is known that traces of water have an adverse effect on both the reaction and the stability of the derivatives. One of the advantages of MTBSTFA is that the *tert.*-BMDS derivatives are much less sensitive to hydrolyzes than e.g. trimethylsilyl-derivatives [24]. The effect of water was evaluated by preparing standard solutions in ethyl acetate containing 0.5, 1.0 or 2.5% (v/v) of water. To 1 ml of these standards in a reaction tube 50 μl of MTBSTFA was added after which the tubes were heated to 75°C for 1 h. The responses obtained were compared with that of a standard containing no water and the differences are given in Table 3. The yields for especially the benzoic acid, dichlorophenol and estradiol decreased with increasing water content. This is likely to be caused by competition between the analytes and the water for reacting with MTBSTFA (ratio water:reagent is 0.28 mmol:0.21 mmol in case of the 0.5%

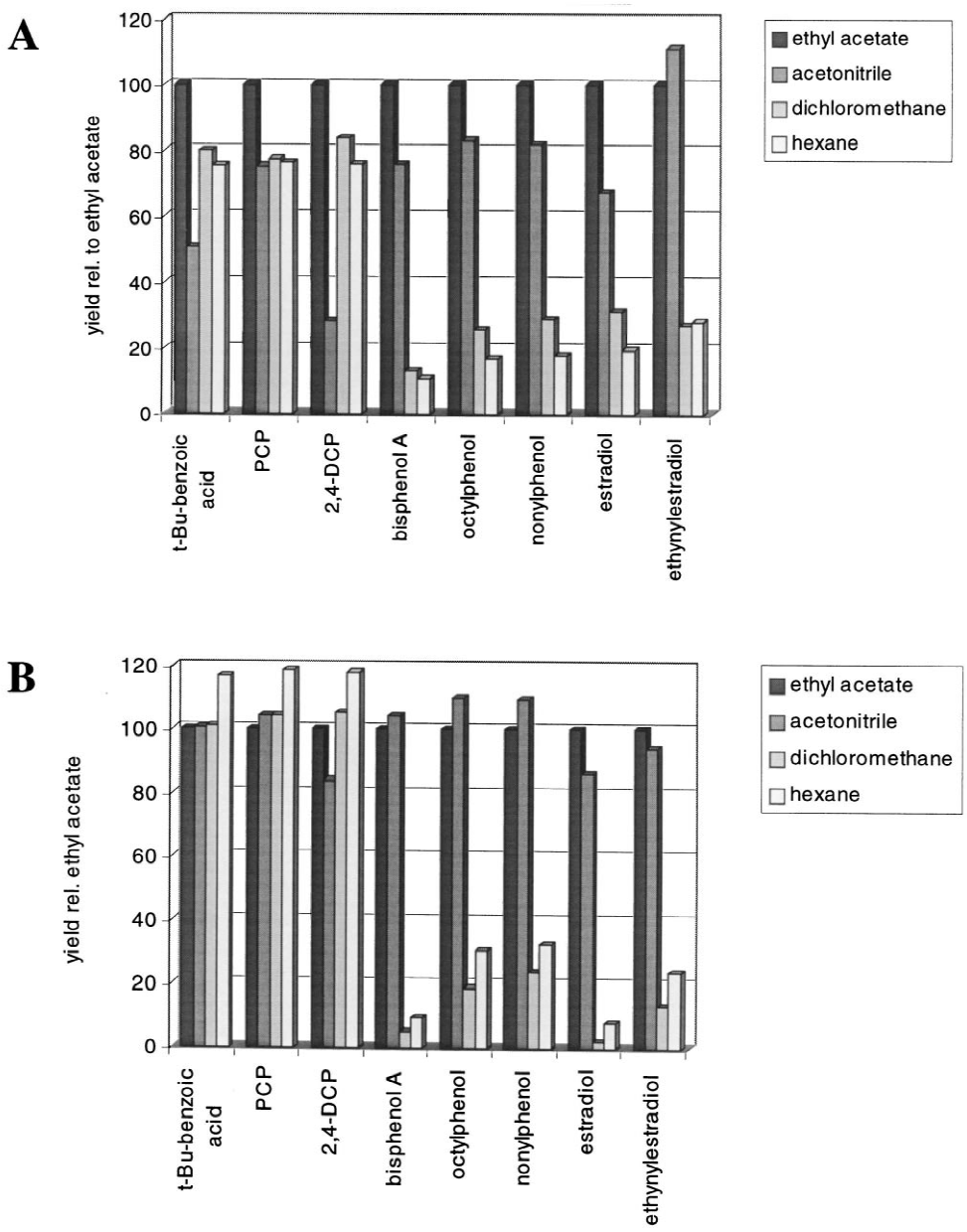

Fig. 3. Effect of solvent on the reaction yield. Reaction yield relative to that obtained for ethyl acetate. Conditions: 1 ml solution, 50 μl of MTBSTFA, temperature 75°C. Reaction time: (A) 30 min; and (B) 3 h.

water in ethyl acetate solution). The responses in case of ethyl acetate containing 2.5% of water (not included in Table 3) could not reliably be calculated as peak deformation and splitting occurred. This was probably due to the less favorable wettability characteristics of the solvent onto the inner wall of the analytical column. When 200 μl of MTBSTFA were added to the tubes the responses were hardly affected, estradiol at 2.5% water being an exception. Furthermore, for the solution with 2.5% water, peaks were less deformed compared to the test where 50 μl reagent was used. Apparently, the water was partly

Table 3
Effect of moisture on derivatization[a]

	Response difference (%) relative to 0% water				
	50 μl reagent added		200 μl reagent added		
	0.5% (v/v) water	1.0% (v/v) water	0.5% (v/v) water	1.0% (v/v) water	2.5% (v/v) water
2,4-Dichlorophenol	−13	−18	−11	−3	−3
tert.-Butylbenzoic acid	−14	−21	−14	−6	−5
Pentachlorophenol	0	2	1	3	1
4-Octylphenol	6	−7	−3	2	2
4-Nonylphenol	9	−5	−1	4	3
Bisphenol-A	3	−11	−4	0	3
17β-Estradiol	−18	−39	2	2	53
17α-Ethynylestradiol	17	9	2	7	13

[a] One milliliter ethyl acetate standard containing 0–2.5% (v/v) water, reaction at 75°C for 1 h.

removed by reaction with MTBSTFA. It can be concluded that traces of water up to 1% have no effect on the derivatization yield as long as an excess of reagent (in mol relative to the amount of water) is added.

Standards derivatized with 200 μl of MTBSTFA and stored in the dark at 4°C were found to be stable for at least 20 days without special precautions for preventing moisture or oxygen to enter the test tube. Our results confirm the data reported by Schoene et al. [24].

3.2.5. Effect of matrix

The aim of this work was to develop a method for surface water. Surface water contains humic substances that may interfere with the derivatization reaction. The amount of humic acids co-extracted with the analytes from water depends on the pH. The lower the pH, the higher the amount of extracted humic substances. Although at this point the optimum pH for extraction of the endocrine disruptors was not yet established, the pH of surface water was adjusted to 2 to simulate a worst case scenario. Matrix was prepared by SPE of 0.5 l of surface water using C_{18} cartridges as described in the Experimental section. The ethyl acetate extract was concentrated to less than 1 ml. Next, a concentrated standard solution was added and the volume was adjusted to 1 ml to give a concentration of approximately 20 μg/ml.

The extracts were derivatized with 50 μl of MTBSTFA, at 75°C over 30 min. While these conditions were fully satisfactory for standards in pure solvent, a dramatic decrease in derivatization yield was observed for most compounds in the real-sample extracts (Fig. 4). Recoveries of over 80% were only obtained for the two most acidic, i.e. reactive, compounds. Increasing the reaction time led to acceptable recoveries for dichlorophenol but for all other compounds the yields were still very low. To examine if the low yields were caused by depletion of the reagent due to competitive reaction with hydroxyl-containing compounds from the surface water, the amount of reagent was increased. The reaction time was kept at 3 h. The results are depicted in Fig. 5. The yield clearly improved with the amount of MTBSTFA supplied and quantitative yields were obtained for all compounds after addition of 400 μl of reagent.

At this point it was not really clear what was the determining factor for obtaining quantitative recoveries, the absolute amount of reagent added, or the concentration of reagent in the extract. Therefore, an additional experiment was conducted in which the surface water extract was further concentrated to 200, 100 μl, or to complete dryness. Derivatization was then carried out using 200 or 400 μl MTBSTFA. The data obtained after GC–MS analysis are shown in Table 4. Recoveries were quantitative when the extract volume was 100–200 μl, irrespective the amount of reagent added. Lower recoveries were obtained for some compounds when the extract was evaporated till dryness. In case of dichlorophenol this was caused by co-evaporation of this compound with the solvent. In case of the other

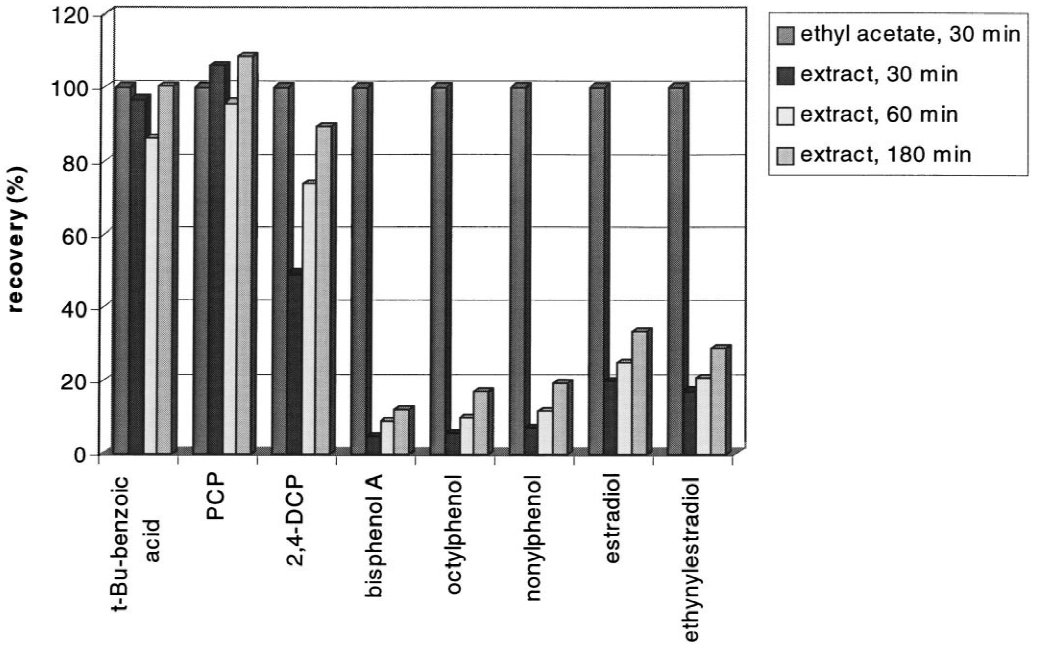

Fig. 4. Effect of matrix on derivatization. Analytes spiked to matrix (Matrix=surface water extracts, SPE after adjustment of pH to 2, 500 ml water⇒1 ml ethyl acetate). Derivatization conditions: 50 μl MTBSTFA, 75°C.

compounds, e.g. bisphenol-A and estradiol, this was caused by difficulties in re-dissolving the derivatives into ethyl acetate. After placing the tubes in an ultrasonic bath for 10 min better recoveries were obtained. To avoid losses, it was decided not to evaporate the solvent completely.

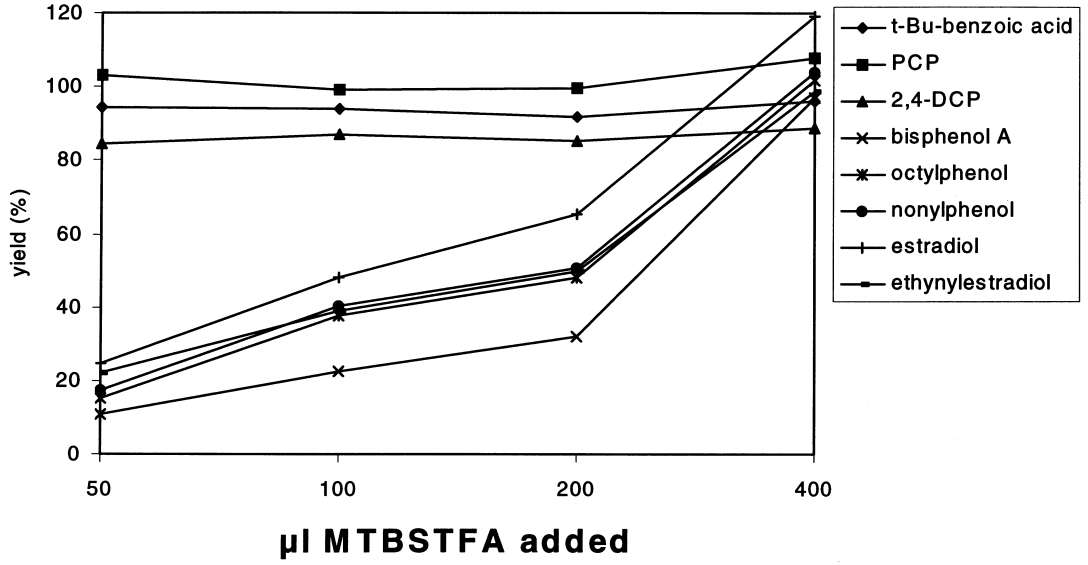

Fig. 5. Effect of amount of MTBSTFA on derivatization yield. Analytes spiked to surface water extracts. Reaction conditions: 75°C, 3 h.

Table 4
Effect of extract volume and amount of MTBSTFA on derivatization yield

	Derivatization yield (%)[a]					
	200 μl reagent			400 μl reagent		
	200 μl extract volume	100 μl extract volume	0 (dry) extract volume	200 μl extract volume	100 μl extract volume	0 (dry) extract volume
2,4-Dichlorophenol	84	87	64	80	86	55
tert.-Butylbenzoic acid	94	94	88	85	95	69
Pentachlorophenol	107	100	95	104	107	80
4-Octylphenol	96	90	70	92	99	68
4-Nonylphenol	98	91	72	97	107	73
Bisphenol-A	91	84	52	99	110	61
17β-Estradiol	121	102	66	132	107	62
17α-Ethynylestradiol	91	87	69	101	110	73

[a] Matrix: 500 ml of surface water, pH 2, extracted using SPE. Derivatization conditions: 75°C, 3 h.

Finally, at the reduced reaction volume and 200 μl of MTBSTFA, the possibility of reducing the reaction time was determined. At 2 h or shorter, quantitative recoveries were not yet obtained, the alkylphenols and bisphenol-A being the most critical compounds (25–60% at 2 h, <20% at 1 h or shorter). It can be concluded that the presence of matrix, humic substances probably being the most significant constituent, diminishes the reaction rate of some of the analytes severely and that high concentrations of reagent (i.e. small reaction volumes) are required in order to obtain quantitative yields within acceptable time.

To summarize, most parameters studied did affect the derivatization yield. Conditions were not very critical for the weak acids (chlorophenols and benzoic acid) but for most other compounds in real-life extracts the following conditions had to be applied for quantitative recoveries: extract volume of 200 μl, 200 μl MTBSTFA, 75°C, 3 h reaction time. These conditions were considered to be acceptable and used throughout the rest of this work.

3.2.6. Stability of tert.-BDMS-derivatives in surface water extracts

Standards in pure ethyl acetate were found to be stable for at least 20 days (see above). As the presence of matrix may effect the stability of the derivatives, the stability was again evaluated over 8 days for all compounds derivatized under optimum conditions in surface water extracts. Extracts were stored in the dark at 4°C, in sealed autosampler vials or in test tubes that were opened every now and then, thereby allowing introduction of moisture and oxygen.

The responses were within the range 80–110% of a freshly derivatized standard in matrix for all compounds from which it can be concluded that extracts are stable for at least 8 days.

3.3. Optimization of extraction

The endocrine disruptors can be extracted by both SPE and liquid–liquid extraction. Based on the optimization of the derivatization, ethyl acetate was selected as desorption/extraction solvent. SPE was preferred over liquid–liquid extraction as it is more time efficient.

3.3.1. Solid-phase extraction

The main parameters to be evaluated for the SPE process were the stationary phase and, because some of the compounds are acidic in nature, the pH of the sample. Two types of cartridges were selected, C_{18} and a PS–DVB polymer phase. Surface water was fortified with the endocrine disruptors at the 0.2–2 μg/l level. To prevent clogging of the cartridges, the water had to be filtered to remove suspended matter. After filtration the pH of the surface water sample (500 ml) was adjusted to pH 2, 4, 5 or 6, and SPE was carried out as described in the Experimental section. Next, derivatization at optimized conditions and GC–MS analysis was performed. The recoveries obtained are shown in Fig. 6 (selected data are

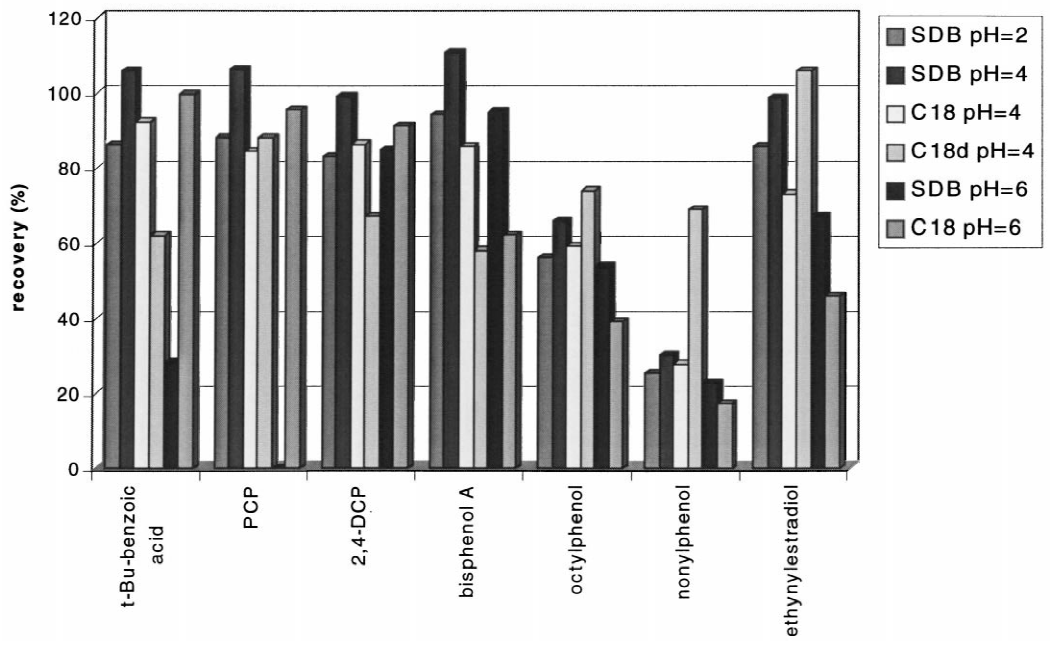

Fig. 6. Optimization of solid-phase extraction. Sample: 500 ml of surface water fortified with endocrine disruptors at the level of 0.2–2 μg/l. SDB, styrene–divinylbenzene polymer cartridge (200 mg, 3 ml); C18, C_{18} cartridge (1000 mg, 6 ml), desorption with 2×2.5 ml of ethyl acetate; C18d, C_{18} extraction disk (47 mm) with glass fiber filter on top, desorption with 3×10 ml of ethyl acetate.

shown). As expected, the pH affected the recoveries of the weak acids *tert*.-butylbenzoic acid and pentachlorophenol. Breakthrough occurred at a pH of 5 or higher for the PS–DVB cartridges. This was not the case when C_{18} cartridges were used. Even at pH of 6, i.e. above the pK_a of 4.7 of pentachlorophenol, recoveries over 90% were still obtained which is probably due to selective interaction of this compound with residual silanol groups of the stationary phase. The stationary phase and pH were not critical for the other compounds, except for the hormones for which, for unknown reasons, lower recoveries were obtained at pH 6.

For octylphenol and, especially, nonylphenol, low recoveries were obtained, irrespective of the pH or the type of cartridge used. As breakthrough was unlikely and desorption with additional ethyl acetate did not improve recoveries the filtration step was studied. To exclude any other SPE related cause, fortified tap water samples were extracted by liquid–liquid extraction with and without prior filtration of the sample. This revealed that the alkylphenols were largely removed by adsorption onto the filter. Filtration over glass fiber instead of paper did not improve recoveries. To solve this SPE related problem the filter has to be desorbed and added to the extract. To avoid a more laborious separate extraction of the filter, a combined filtration/SPE set up was tested. To this end a SPE disk was used instead of a cartridge, and a glass fiber filter was placed directly on top of the disk. This approach proved to be more practical then SPE cartridges with filter cartridges mounted on top of them. In addition, the disk set up did not involve tubing for sampling of the water which could be another possible cause of losses of non-polar analytes. Furthermore, the larger area (47 mm I.D.) of the disk relative to the cartridge improved the sampling time, although this was partly annuled by the larger desorption volume which prolonged the time for evaporative concentration of the extract. The approach was quite successful as now recoveries of 60% or higher were obtained for all compounds including the alkylphenols. The combined filtration/SPE set up with the glass fiber filter resulted in somewhat reduced recoveries for the benzoic acid and bisphenol-A, probably caused by

adsorption to and incomplete desorption from the glass fiber filter. The use of other types of filters may improve this but no attempts were made here.

To summarize, with SPE acceptable recoveries can be obtained for all endocrine disruptors from this study at pH 4, using either C_{18} or PS–DVB as stationary phase. The alkylphenols are prone to losses during filtration of the water sample and the SPE design should be such that possibly adsorbed analytes are extracted from the filter again.

3.3.2. Liquid–liquid extraction

As with SPE some problems were encountered with the required filtration step, liquid–liquid extraction was also examined. The water samples were extracted twice with 100 ml of ethyl acetate as organic solvent and only the effect of the pH was evaluated. This was done for Milli-Q water as well as surface water. In case of Milli-Q water, in the pH range 2–6, recoveries better than 75% were obtained for all compounds except for ethylnylestradiol (ca. 60%). The results obtained for surface water are shown in Fig. 7. *tert.*-Butylbenzoic acid was quantitatively extracted up to a pH of 6, after which the extraction efficiency dropped to 20% at pH of 8. A bit surprisingly, pentachlorophenol was still quantitatively extracted at pH 8 (i.e. well above its pK_a).

Two unexpected observations were made: (i) a decrease in recoveries for the alkylphenols and bisphenol-A when adjusting the pH below five; and (ii) lower response and tailing peaks for the two hormones. One explanation for this could be that the extract obtained after liquid–liquid extraction is different (e.g. more 'dirty') then a SPE extract. This may affect the derivatization (note that optimization was performed with SPE extracts) and the GC analysis. To examine the first mentioned item, surface water was extracted at pH of 2 and 6. The analytes were added to the extracts after which derivatization and GC analysis were carried out. Recoveries for extracts obtained at pH 2 and 6 were 71–110 and 94–110%, respectively, demonstrating that there were no problems during the derivatization step. The response and peak shape of the hormones, however, did not improve, indicating the presence of a GC-related problem. This was confirmed by the fact that response and peak shape of the hormones could be restored by one or two injections of clean

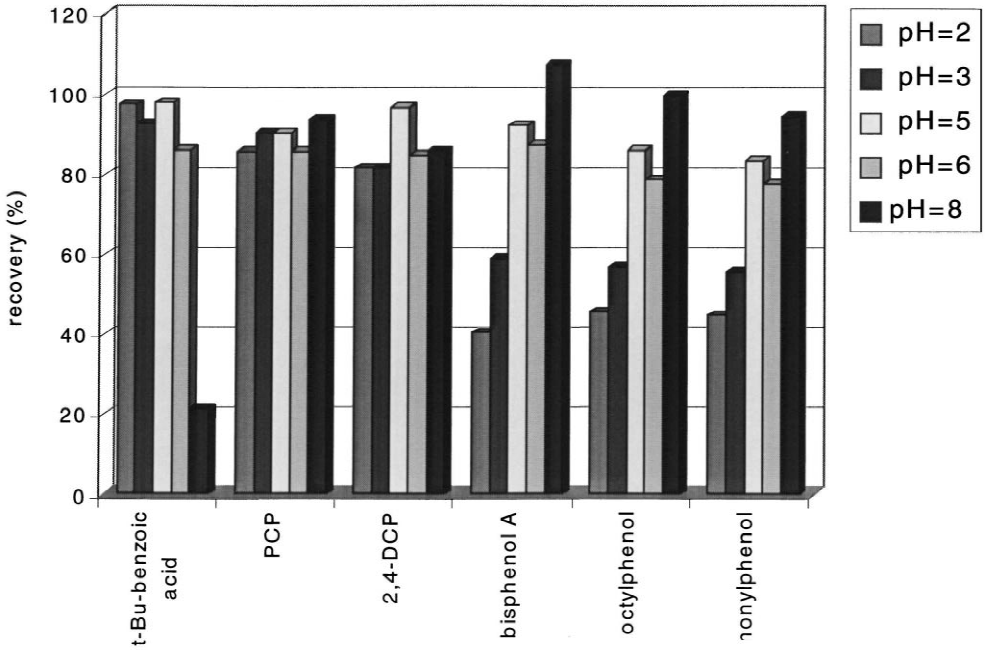

Fig. 7. Effect of pH on liquid–liquid extraction. Sample: 500 ml of surface water fortified with endocrine disruptors at the level of 0.2–2 μg/l. Extraction: twice with 100 ml of ethyl acetate.

Table 5
Analytical performance characteristics of determination of endocrine disruptors in surface water[a]

	Liquid–liquid extraction, (pH 6) $n=6$, level: 0.2 μg/l		SPE, C_{18} disk (pH 4) $n=4$, level: 0.05–0.12 μg/l		LOD ($S/N=3$) (ng/l)
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	
2,4-Dichlorophenol	109	14	67	15	4
tert.-Butylbenzoic acid	103	8	62	10	4
Pentachlorophenol	115	8	88	6	6
4-Octylphenol	115	11	74	10	6
4-Nonylphenol	114	9	69	10	6
Bisphenol-A	117	6	58	10	5
17β-Estradiol	–	–	–	–	300
17α-Ethynylestradiol	–	–	106	16	50

[a] Sample: surface water, concentration factor=500, 2 μl splitless injection; MS, full scan aquisition.

ethyl acetate. Apparently, the response for the hormones, that still have one underivatized hydroxyl group, deteriorates when injected together with matrix obtained after liquid–liquid extraction. A pH of 5–6 was considered optimal for liquid–liquid extraction.

3.4. Analytical performance

Based on the above discussion on extraction, SPE is preferred when hormones are to be included in the method, while otherwise liquid–liquid extraction is preferrable because of the better extraction efficiency. Therefore, for both options the accuracy and repeatability were determined. The results are given in Table 5. Acceptable recoveries and RSDs were obtained for almost all analytes and for both extraction methods.

Liquid–liquid extraction resulted in better recoveries and slightly better repeatabilities. The limits of detection (LOD; $S/N=3$) as determined from the data obtained after SPE of surface water samples spiked at 20 ng/l (higher for hormones) and full scan aquisition, were in the 4–6 ng/l range for all compounds except for the hormones. Due to the presence of one underivatized hydroxyl group, their response was still dependent on the matrix and the condition of the analytical column. This limited the application of the method for hormones to levels that, in surface water, are higher then expected in real samples. Sensitive analysis was possible for all other compounds in a routine manner, i.e. ca. 100 injections could be performed without deterioration in performance.

The linearity of the MS response vs. concentration of analytes was good (regression coefficient better than 0.996) for all compounds, except pentachlorophenol (r.c. 0.983), over the range LOD to 1 μg/l.

In Fig. 8 typical chromatograms obtained after analysis of surface water fortified at low level are shown. The peaks of the hormones are slightly tailing while sharp peaks, good selectivity, and excellent sensitivity was obtained for all other compounds.

4. Conclusions

Derivatization of a number of hydroxyl-containing endocrine disruptors, including *tert.*-butylbenzoic acid, 4-*n*-alkylphenols, chlorophenols, bisphenol-A and two hormones, enables the sensitive determination of these compounds by gas chromatography. MTBSTFA was found to be a suitable agent for substitution of the hydroxyl groups. For the hormones estradiol and ethynylestradiol, however, only one of the two hydroxyl group was substituted.

For all compounds, except the weak acids like *tert.*-butylbenzoic acid and pentachlorophenol, the derivatization yield was affected by most parameters studied. Especially the presence of matrix had a strong effect. Reduction of extract volume to <200

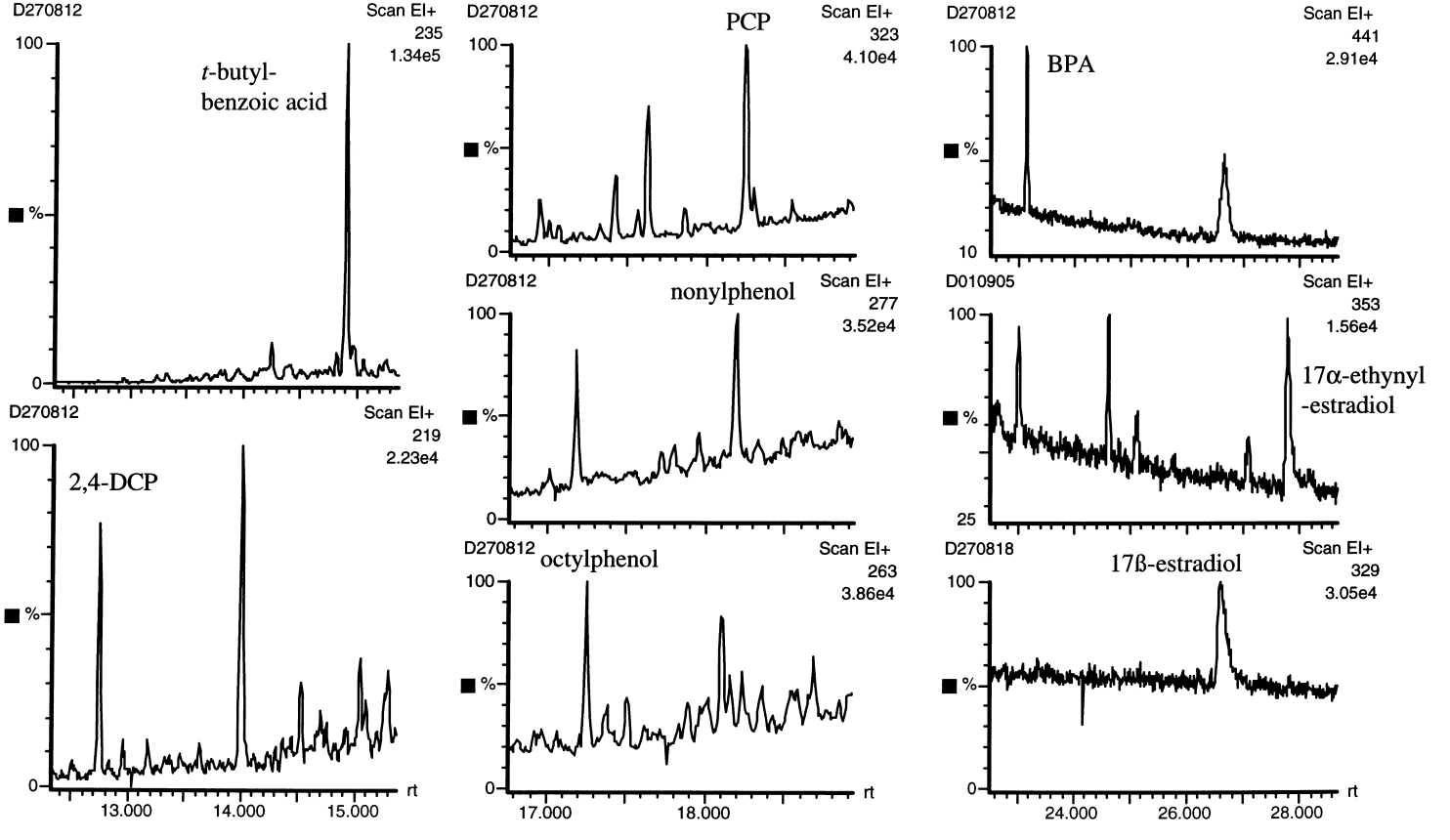

Fig. 8. Representative extracted ion chromatograms of endocrine disruptors obtained after GC–MS analysis. Level of fortification: estradiol 0.5 μg/l, ethylnylestradiol 0.1 μg/l, others 0.02 μg/l. 2,4-DCP, 2,4-dichlorophenol; PCP, pentachlorophenol; BPA, bisphenol-A; rt, retention time in minutes.

μl, addition of excess of reagent (200 μl), an elevated temperature (75°C) and a reaction time of 3 h were essential for obtaining quantitative recoveries for all compounds in real-life extracts.

Both SPE (at pH 4) and liquid–liquid extraction (at pH 5–6) were found to be suited for extraction of water samples. In case of SPE, care had to be taken to prevent losses of octyl- and nonylphenol during the filtration step that was required to prevent clogging of the SPE material.

The method proved to be suited for routine application to surface water analysis down to the level of 4–6 ng/l for all compounds except the hormones. For the latter, due to the fact that these bifunctional compounds were only mono-substituted, detection limits were restricted to 50–300 ng/l.

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