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Application of solid-phase extraction coupled with freezing-lipid filtration clean-up for the determination of endocrine-disrupting phenols in fish

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ABSTRACT

An analytical method has been developed for the determination of endocrine-disrupting phenols (eight alkylphenols and bisphenol A) in fish samples. The extraction of nine phenols from fish samples was carried out by ultrasonification. After the extraction, high levels of lipids were removed by freezing-lipid filtration instead of the traditional methods of column chromatography or saponification. During freezing-lipid filtration, about 90% of the lipids were eliminated without any significant loss of phenolic compounds. For further purification, hydrophilic–lipophilic balanced copolymer (HLB) sorbent with a poly(divinylbenzene-co-*N*-vinylpyrrolidone) phase and Florisil-solid-phase extraction (SPE) cartridges were used to eliminate the remaining interferences. Silyl-derivatization, with *N*,*N*′-methyl-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA), was applied to enhance the sensitivity of detection of phenolic compounds. Quantification was performed by gas chromatography/mass spectrometry (GC/MS)-selected ion monitoring (SIM) mode, using deuterium-labeled internal standards. Spiking experiments were carried out to determine the recovery, precision and detection limit of the method. The overall recoveries ranged between 70 and 120%, with relative standard deviations of 3–17% for the entire procedure. The detection limits of the method for the nine phenols ranged from 0.02 to 0.41 ng g^{-1}. The method provided simultaneous screening and accurate confirmation of each phenol when applied to biological samples.

1. Introduction

Alkylphenols and bisphenol A have been of increasing concern, as they are suspected endocrine disruptors. In particular, these compounds have been reported to show estrogenic activity in both *in vitro* and *in vivo* screening systems [1–4]. Alkylphenols have been widely used in the chemical industries as herbicides, detergents and synthetic resin products. Also, bisphenol A has been widely used as a material for the production of epoxy resins, phenol resins, polycarbonates, polyesters and lacquer coatings on food cans [5,6]. Furthermore, alkylphenols and bisphenol A have been detected, in

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significant concentrations, in marine and freshwater habitats around the world [7–9]. Continued development of analytical methodologies is needed for the accurate determination of alkylphenols and bisphenol A in both environmental and biological samples.

Many analytical methods have been reported for the measurement of alkylphenols and bisphenol A to determine not only their presence but also their concentrations in samples with high precision and accuracy. In particular, the analysis of phenols present at sub-nanogram levels in biological samples requires extensive sample extract clean-up procedures prior to their introduction into sensitive instruments. Moreover, the clean-up procedure for fish tissue is complicated due to the co-extraction of large quantities of lipids compared with other environmental samples. In general, the lipid content of fish tissue is about 15% of the net mass, and is composed of several glycerolipids with high molecular weights which can significantly reduce the analytical performance of GC/MS due to the accumulation of residues in the injection port, column and ion source [10–12]. Several approaches have been developed to eliminate these co-extracted lipid interferences, including liquid–liquid partitioning [13], gel permeation chromatography [14–16], column chromatography [17] and multiple clean-up methods [18].

As alternative clean-up methods, acidic or basic treatments, such as saponification, have typically been used for the elimination of lipid components [19,20]. These destructive clean-up methods have the advantage of simplicity and economy, but they are time consuming and toxic chemicals are required for the partitioning procedures. Recently, solid-phase extraction (SPE), using cartridges filled with various sorbents, has been extensively used due to the advantages of rapid and simple manipulation as well as the small amounts of solvent consumption compared with conventional column chromatography. In particular, Florisil, silica and alumna have been used as cartridge sorbents for the separation of phenolic compounds from co-extracted contaminants [21]. In some cases, solvent partitioning has been applied to remove oily matrices from extracts [22]. However, this procedure may cause the loss of some analytes due to the formation of emulsions and their different solubilities in organic solvents.

Recently, freezing-lipid filtration has been developed to eliminate large amounts of lipids extracted from biota samples. This method has been applied for the determination of chlorinated pesticides [23], anabolic steroids and synthetic hormones [24], as well as polychlorinated dibenzo-*p*-dioxins and furans (PCDDs/Fs) [25] in biological samples. The large amount of lipids extracted from biological samples can easily be removed through freezing lipid in the cold extract, followed by filtration. Thus, this freezing-lipid filtration method has several advantages over conventional methods with respect to effective sample clean-up and the relatively easy combination with SPE clean-up.

For the detection of various phenols in environmental media, liquid chromatography, with UV [26], fluorescence [27], and electrospray ionization (ESI) [28] and atmospheric pressure chemical ionization (APCI) [29] mass spectrometry have commonly been used. Also, gas chromatography of phenolic compounds, with flame ionization detection (FID) [30] and mass spectrometry [31,32], has been widely used. However, chemical derivatization techniques in GC and GC/MS analysis are inevitably required for the detection of phenols in complex matrices and for improvement of their chromatographic properties. Various derivatization techniques, such as alkylation [33], acylation [34], silylation [35] or pentafluorobenzoate derivatization [36], have been reported. Among them, the silylation technique has been popular due to its high sensitivity and stability in GC and GC/MS analysis.

The aim of this study was to apply a new method, freezing-lipid filtration, followed by SPE cartridge clean-up and quantification using GC/MS for the determination of endocrine-disrupting phenols in fish tissue. In SPE, the elution profile and adsorbents were optimized for effective clean-up. Silyl-derivatization and quantification, using deuterium-labeled internal standards, were performed to provide sufficiently sensitive and precise analytical results. With this method, over 90% of lipids had been removed after freezing-lipid filtration and the main interferences such as fatty acids have been eliminated to separate phenolic compounds during the SPE procedure. Therefore, this method can be applied to the monitoring of endocrine-disrupting phenols in fish with respect to the South Korean regulations.

2. Experimental

2.1. Standards and reagents

Pure standards of nine phenols were purchased from Acros Organic (Morris Plains, NJ, USA). Deuterium-labeled internal standards were purchased from C/D/N Isotopes (Pointe-Claire, QC, Canada). A standard mixture of nine phenolic compounds (100 $\mu g\,mL^{-1}$) in acetone, and working standard solutions (0.5–50 $\mu g\,mL^{-1}$), were prepared and then stored at −20 °C prior to use. Fixed amounts of three deuterium internal standards, at concentrations of 10 $\mu g\,mL^{-1}$, were prepared and spiked in the experiments.

Organic solvents (hexane, methanol, methyl *tert*-butyl ether (MTBE), and acetone) of pesticide residue analytical grade were purchased from J.T. Baker (Philipsburg, NJ, USA). Anhydrous sodium sulfate, used as a drying agent, was purchased from Merck (Darmstadt, Germany). All glassware was cleaned with laboratory detergents, and no plastic products were used so as to avoid contamination. The SPE cartridges containing Oasis® HLB, C8 (500 mg, 6 mL), Florisil, and silica (5 g, 20 mL) were obtained from Waters (Milford, MA, USA).

The silyl-derivatization reagents, *N*,*N*′-methyl-(trimethylsilyl) trifluoroacetamide (MSTFA) and *N*,*N*′-methyl-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) were obtained from Pierce (Rockford, IL, USA).

2.2. Samples

Pooled filleted mackerel samples, weighing about 500 g, were homogenized and then stored below −20 °C pending analysis. Aliquots of the samples, containing none of the target compounds, were used for the spiking experiments. The spiked samples were then stored in tightly closed amber-colored glass bottles at room temperature for 24 h.

2.3. Sample extraction and delipidation

A 10 g ground sample, spiked with 100 ng deuterium labeled internal standards, was added to 30 g of powered anhydrous sodium sulfate. Extraction was carried out by sonication for 30 min with 70 mL of methanol. The extract was filtered and dried to determine the lipid content gravimetrically according to the EPA method. [37] The extract was then dissolved in 50 mL of methanol, and stored at −24 °C for 40 min to freeze the lipids. Most of the lipids were suspended as clusters and were easily removed via filtration. Lipid removal (%) was calculated from the residue of lipid after freezing-lipid filtration. The detailed freezing-lipid filtration procedure has been described in our previous study [23]. The filtered extract was concentrated for application to the SPE procedure.

2.4. SPE clean-up

The dried extract was redissolved in 2 mL of *n*-hexane for application to the SPE clean-up. For an HLB-SPE, the cartridge was sequentially pre-conditioned with 6 mL of *n*-hexane and 6 mL of a methanol/MTBE (30:70, v/v) mixture. After the extract had been loaded onto the SPE cartridge, the cartridge was washed with 10 mL of *n*-hexane and 1 mL of a methanol/MTBE mixture, and then 2–4 mL of a methanol/MTBE mixture was eluted for collection. The eluent collected from the HLB-SPE clean-up was loaded onto the Florisil SPE cartridge which had been pre-conditioned with *n*-hexane and a *n*-hexane/acetone (30:70, v/v) mixture, and washed with *n*-hexane (10 mL) and 6 mL of the *n*-hexane/acetone mixture. The phenolic compounds were collected with the elution fraction, 7–15 mL of the *n*-hexane/acetone mixture as shown in Fig. 1. The eluent was evaporated to dryness under a gentle stream of nitrogen for the derivatization step.

2.5. Derivatization

The silyl derivatization was carried out in the test tubes in which the sample had been dried following the SPE procedure. To compare the stability and sensitivity of the silylated phenol derivatives, MSTFA and MTBSTFA were used as silylation agents.

To the dried eluent, 100 μL of derivatizing reagents (MSTFA, MTBSTFA) was added and vortex-mixed for 30 s. The reaction

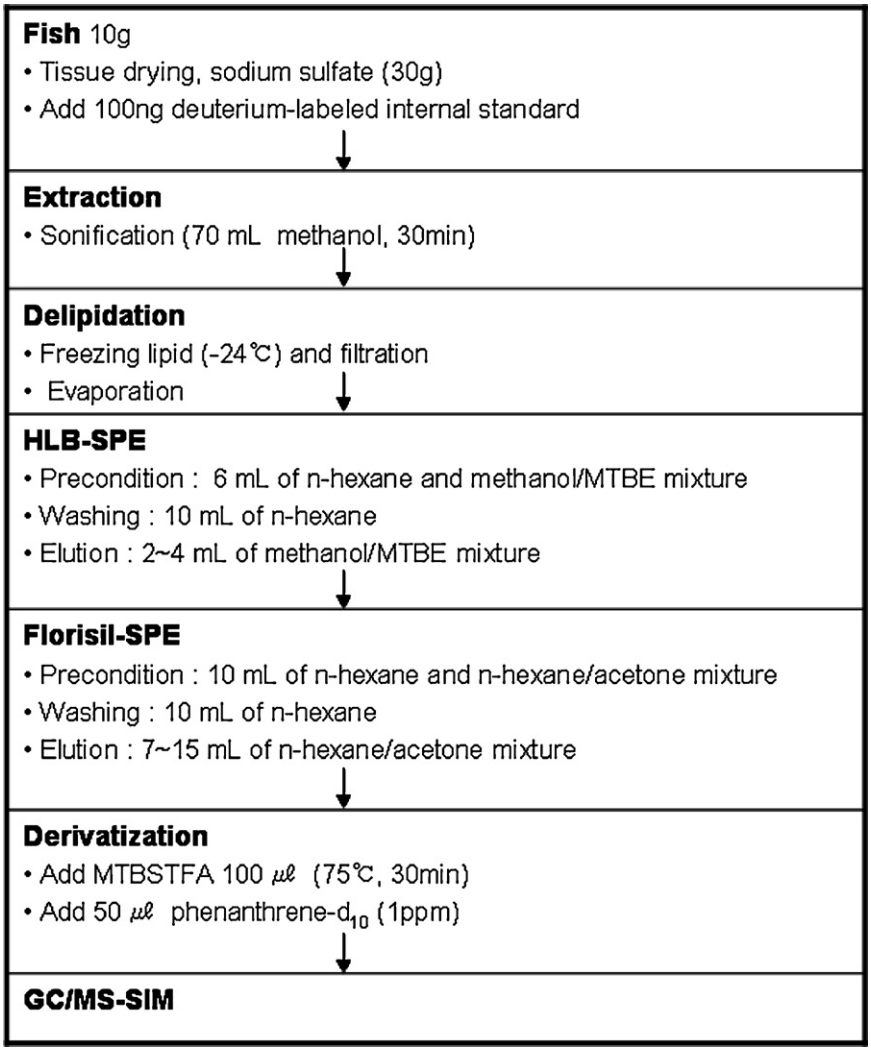

Fig. 1 – Analytical procedure for the determination of endocrine-disrupting phenols in fish tissue samples.

mixture was heated at 75 °C for 30 min. After the silyl derivatization, the resulting solution was left at room temperature. To test the reaction yield and stability, 50 μL of phenanthrene-d_{10} was added as an internal standard. Fig. 1 shows the analytical procedure for the determination of the endocrine-disrupting phenols in fish samples.

2.6. 2.6 GC/MS analysis

GC/MS analysis was performed with an Agilent 6890 Plus gas chromatograph, equipped with a 5973N mass selective detector quadrupole mass spectrometer system (Palo Alto, CA, USA). The DB-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, 5% diphenyl—95% dimethylsiloxane phase) was obtained from J&W Scientific (Folsom, CA, USA). The GC oven temperature was maintained at 100 °C for 1 min, and then ramped to 200 °C at 3 °C min^{-1}, and maintained for 1 min, then to 280 °C at 20 °C min^{-1} and maintained for 5 min. The sample was injected in the split mode, at a splitting ratio of 1:10. The temperatures of the GC injection port and MS interface were set at 260 and 280 °C, respectively. The mass selective detector was run in the electron impact (EI) mode, with an electron energy of 70 eV. The instrumental parameters were set at the following values: 300 μA filament emission current and multiplier voltage of 2200 V. The mass spectrometer was operated in the full scan mode between 50 and 550 amu. For monitoring and a confirmation analysis, the selected ion monitoring (SIM) mode was used, with the dwell time of each ion set at 50 ms. To improve the sensitivity, selected ions in the SIM mode were divided into three groups, guided by their retention times. The selected ion groups in the SIM mode are listed in Table 1. All phenolic compounds were identified by their retention times and specific ions, and quantified using the internal standard method.

3. Results and discussion

3.1. Extraction and delipidation

The sample was homogenized and extracted with a suitable solvent to remove the bulk of the sample matrix and extract the phenolic compound residue into the solvent. Both the selection of solvent and extraction method can be critical for the satisfactory recovery of phenols from the sample matrix. In this study, methanol was used as the ultrasonic extraction solvent for the phenolic compounds from the spiked fish tissue, since methanol has shown good solubility for phenols, but less solubility for non-polar compounds. Under these extraction conditions, the overall extraction yield of phenolic compounds was above 95%, due to the good solubility of methanol for phenolic compounds. Moreover, the extraction time can be greatly reduced using ultrasonic extraction.

During the extraction of the phenols from the fish tissue, high-molecular weight lipids, including triglycerides, as well as the extractable polar compounds in methanol were co-extracted, although lipids are less soluble in methanol. It is difficult to pre-treat the sample extracts for the selective extraction of the phenols of interest, as well as remove the lipid interferences from the extract. Normally, several clean-up steps, including solvent partitioning and column clean-up, are required to remove the lipid materials. In some cases, destruction of the lipid matrix was conducted using saponification to effectively eliminate the fatty matrix [38]. In this study, to eliminate the lipids extracted from biological samples, a solvent partition method was initially applied due to its ease of use. However, hexane–methanol partitioning had relatively poor recoveries for *n*-alkylphenols, giving less than about 60% yields.

In another trial, freezing-lipid filtration was applied for the removal of the lipid materials extracted from fish tissue.

Table 1 – Selected ions (*m/z*) and retention times (min) of silylation derivatives

Compound	TMS			TBDMS		
	RT (min)	Quant. ion (*m/z*)	Confirm ion (*m/z*)	RT (min)	Quant. ion (*m/z*)	Confirm ion (*m/z*)
Group 1						
4-*tert*-Butyl phenol	10.51	151	207	18.28	207	222
4-*n*-Butylphenol	12.82	179	222	20.92	264	207
4-*n*-Pentyl-d_{11}-phenol	15.89	181	247	24.08	232	289
4-Pentylphenol	16.19	179	236	24.36	221	278
4-*n*-Hexylphenol	19.76	179	250	27.86	235	292
4-*tert*-Octyl phenol	20.30	207	278	28.35	249	320
Phenanthrene-d_{10}	24.99	188		24.99	188	
Group II						
4-*n*-Heptyl phenol	23.31	179	264	31.20	249	306
Nonylphenol	23.73/23.95/24.12/24.27	207	292	31.47/31.79/31.94/32.11	249	334
4-Octylphenol	26.75	179	278	34.47	263	320
4-*n*-Nonylphenol-2,3,4,5-d_4	30.07	183	296	36.40	281	338
Group III						
Bisphenol A-d_{16}	36.97	368	386	40.99	452	470
Bisphenol A	37.05	357	372	41.07	441	456

The clean-up of phenolic compounds from lipids using the freezing-lipid filtration method is based on solidifying the lipid in a solvent at −24 °C, with just filtration of the freezing lipids. Most of the lipids are suspended as white clusters in methanol at low temperature due to their low melting points, whereas the phenolic compounds are still soluble in cold methanol. From our previous results [23], acetonitrile was shown to be the best solvent for freezing lipids at low temperature. However, methanol was used as the extraction solvent in this study for the delipidation at −24 °C due to the lower extraction efficiency of phenolic compounds by acetonitrile. During the freezing-lipid filtration, about 90% of the lipids in the extract could be eliminated without any significant loss of phenolic compounds.

3.2. *Solid-phase extraction*

After the freezing-lipid filtration, the extract still contained about 10% of the lipids; a maximum of 100 mg per 10 g fish tissue sample. In particular, polar lipids, such as fatty acids, phospholipids and cholesterol are co-present in relatively high amounts compared with the sub-microgram levels of phenols in methanol. These compounds could interfere with the phenolic compounds in the GC/MS analysis. Therefore, an additional clean-up step was required to reach the sub-ng g^{-1} level of quantification in the analysis of biological samples.

Several SPE cartridges were tested for their convenience and simple clean-up methods for the elimination of the remaining lipids following freezing-lipid filtration. The HLB-SPE cartridges (500 mg, 6 mL) were used to remove the triacylglycerol and glycerophosphocholine residues. The HLB-SPE is known to effectively remove both hydrophilic and lipophilic interferences during the clean-up procedure [39]. In addition, there is no need for a drying step with an HLB cartridge. Fig. 2 shows the elution patterns with the HLB cartridge when the methanol/MTBE mixture (v/v, 30:70) (Fig. 2A) and methanol (Fig. 2B) were used as the elution solvents. Most of the phenolic compounds were eluted with 4 mL of eluent on the HLB cartridge with the methanol/MTBE mixture elution. On the other hand, the elution volume increased to 7 mL due to the stronger retention of some of the phenols on the HLB cartridge when methanol was used as the elution solvent. Therefore, the methanol/MTBE mixture was selected for the HLB-SPE clean-up.

Although freezing-lipid filtration, combined with HLB-SPE clean-up, has been shown to be an effective method for the elimination of lipids, significant amounts of fatty acids and cholesterol still remained in the sample extract. These compounds could significantly interfere with the phenolic compounds in the GC/MS analysis. For further clean-up, Florisil and silica sorbent (5 g, 20 mL) cartridges were used to compare the removal efficiency of these interferences. An *n*-hexane/acetone mixture (v/v, 30:70) was used as the elution solvent for both of the cartridges. Fig. 3 shows the elution patterns of the phenolic compounds, eluted with the *n*-hexane/acetone mixture, from the silica (Fig. 3A) and Florisil-SPE cartridges (Fig. 3B). The elution patterns of the phenolic compounds from both the SPE cartridges were similar, with the exception of bisphenol A. The elution of phenolic compounds from the Florisil-SPE cartridge was earlier than from the silica-SPE cartridge.

Total ion chromatograms of blank fish tissue samples, purified using the silica- and Florisil-SPE cartridges, are shown in Fig. 4. With silica-SPE, several interferences were detected in the total ion chromatogram (upper trace in Fig. 4). The main interferences were C_{16}–C_{20} fatty acids and cholesterol (retention times range from 34 to 42 min). Moreover, the baseline was significantly high in this range of retention times, with insufficient sensitivity for nonylphenol, bisphenol A and its deuterium labeled internal standard. Conversely, when the extract was purified using the Florisil-SPE, significant amounts of fatty acids were effectively eliminated, as shown in the lower trace of Fig. 4. Although some fatty acids were still observed in the GC/MS-scan mode after the Florisil-SPE step, the appearance of these interferences could be greatly reduced by the selection of specific ions in the GC/MS-SIM mode. Therefore, the extract could be successfully purified and analyzed by freezing-lipid filtration, HLB-SPE, Florisil-SPE and GC/MS-SIM.

3.3. *Silyl derivatization*

Even though polar stationary phases, such as “wax”, are widely used in some polyethylene glycol (PEG) columns for the separation and detection of phenols. Bisphenol A could not be detected owing to the limited column temperature [40]. Derivatization of phenolic compounds is required for their detection at sub-ng g^{-1} levels by GC–MS analysis. The interac-

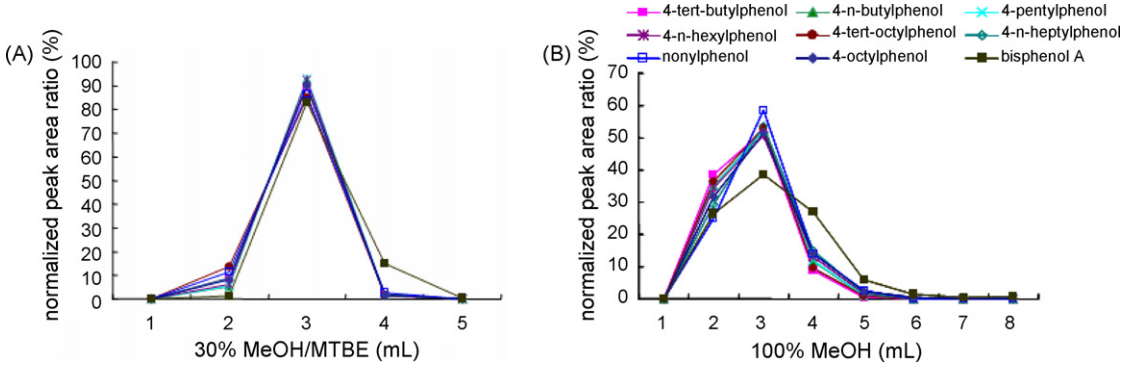

Fig. 2 – Elution patterns of endocrine-disrupting phenols from the HLB cartridge, according to elution solvents: (A) 30% MeOH/MTBE (500 mg HLB) and (B) 100% MeOH (500 mg HLB).

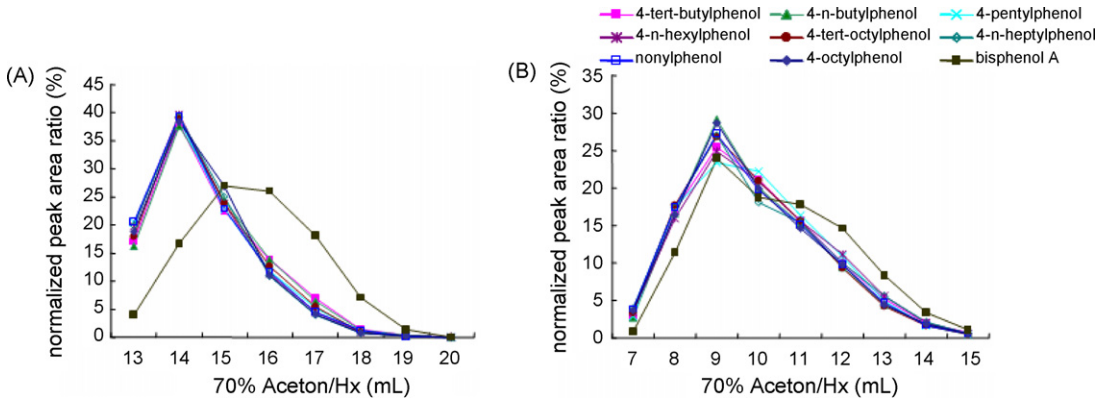

Fig. 3 – Elution patterns of endocrine-disrupting phenols from silica (A) and Florisil (B) cartridges, eluted with 70% acetone/Hx.

tion between the hydroxyl group in the phenol ring and active sites in the GC system, such as the injection port and column, could be greatly minimized when silyl-derivatization was used. In addition, another advantage of silyl-derivatization is the production of diagnostic ions for the characterization of their identities in the mass spectra. The characteristic ions of silyl-derivatives are the $[M\text{-}15]^+$ ion for the trimethylsilyl (TMS) derivatives, and the $[M\text{-}57]^+$ ion for the *tert*-butyldimethylsilyl (TBDMS) derivatives. These ions, which appeared as base peaks in the mass spectra, can be used as quantification ions for phenol derivatives in the GC/MS-SIM mode.

In this study, TMS and TBDMS derivatizations by MSTFA and MTBSTFA, respectively, were applied to compare the silylation reaction yields and stability of the derivatives. Both reagents showed good reaction yields under the derivatization conditions described in the Experimental section. In the GC/MS-SIM mode, there was no significant difference in the responses between the TMS and TBDMS derivatives; however, there was a great difference in their storage stabilities. The amounts of phenol–TMS derivatives decreased to 60% after 8 days due to their easy hydrolysis. Conversely, the TBDMS derivatives remained stable, even after 8 days. This observation was in good agreement with the results from another study [41]. In addition, the retention times of TBDMS derivatives are generally longer than those of TMS derivatives, resulting in their successful separation from the earlier eluted interferences. Thus, MTBSTFA was used as the derivatization agent in this study.

The relative response factors (RRFs) of the phenol–TBDMS derivatives were calculated using the peak area ratio relative to phenanthrene-d_{10} as internal standard. Although not shown here, the RRFs of phenol–TBDMS derivatives were 2.1–28.9 times greater than the free phenols. In particular, the RRF of the bisphenol A–TBDMS derivative can be significantly increased by over 28 times. In the case of nonylphenol, four isomers of the nonylphenol–TBDMS derivative were observed in high abundance, but free nonylphenol was observed as several isomer peaks with low abundance. The TBDMS-derivative was not only able to increase the analyte detectability, but also decreased the uncertainty of quantification.

3.4. *Method application*

Deuterium-labeled internal standards and phenolic compounds were spiked into the control fish sample, and then extracted, purified, derivatized, and analyzed by the method

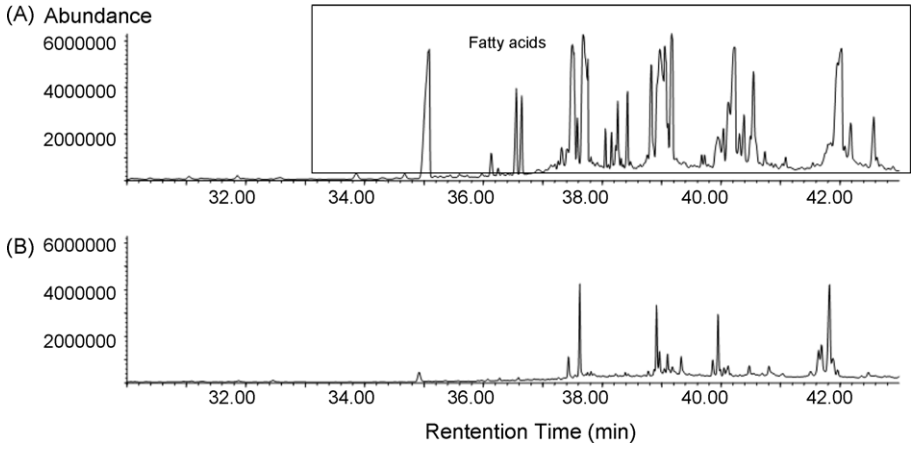

Fig. 4 – Total ion chromatograms of fish extracts, cleanup with 5 g silica-SPE (A) and Florisil-SPE (B), after HLB cartridge clean-up in the GC/MS scan mode.

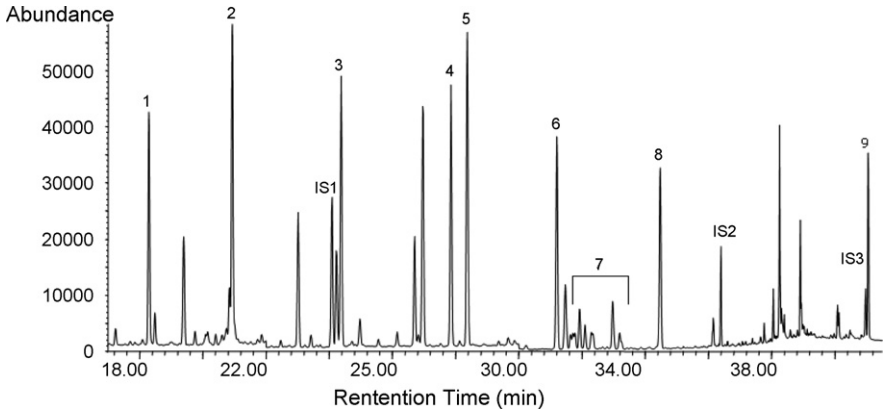

Fig. 5 – SIM chromatogram of TBDMS derivatized endocrine-disrupting phenols in a spiked fish sample at 10 ppb level. Peak identities as follows: (1) 4-*tert*-butylphenol-OTBDMS, (2) 4-*n*-butylphenol-OTBDMS, (3) 4-pentylphenol-OTBDMS, (4) 4-*n*-hexylphenol-OTBDMS, (5) 4-*tert*-octylphenol-OTBDMS, (6) 4-*n*-heptylphenol-OTBDMS, (7) nonylphenol-OTBDMS isomers, (8) 4-octylphenol-OTBDMS, and (9) bisphenol A-(OTBDMS)$_2$ IS1. 4-*n*-pentyl-phenol-d$_{11}$-OTBDMS, IS2. 4-*n*-nonylphenol-d$_4$-2,3,5,6-OTBDMS, and IS3. bisphenol-d$_{16}$-(OTBDMS)$_2$.

developed. A typical total ion chromatogram of the phenol compounds extracted from the spiked fish is given in Fig. 5. No significant interferences were observed in the total ion chromatogram. The ratios of the peak areas of TBDMS derivatized phenols and the corresponding internal standards were determined. The calibration curves were generated using a least-squares linear regression analysis ranging between 0.5 and 50 ng g^{-1}. The correlation coefficients for TBDMS derivatized phenols were higher than 0.991.

The detection limit was determined as the lowest amount of analyte corresponding to a signal/noise ratio of 3. In the GC/MS-SIM analysis, the base peaks of the derivatives, *m*/z 151 for 4-*tert*-butylphenol, *m*/z 264 for 4-*n*-butylphenol, *m*/z 249 for nonylphenol and *m*/z 441 for bisphenol A were used as the quantification ions for the lower detection limit. For the remainder of the TBDMS derivatized phenolic compounds, the [M-57]$^+$ ion was the base peak, as selected in the SIM mode. In the SIM mode, the *m*/z 207 ion was selected for monitoring the butylphenol derivatives. However, the abundance of the *m*/z 207 ion should be checked carefully at background levels, as it could originate from column bleeding, providing false-positives or overestimation of the corresponding phenols.

Method detection limits (MDLs) and analyte recoveries for the 1 and 10 ng g^{-1} spiked fish sample are listed in Table 2. MDLs and recoveries were measured by analyzing seven replicate samples spiked with phenolic standards per 10 g of control fish samples. The recovery of the phenolic compounds ranged from 70 to 120%, with relative standard deviations (R.S.D.) ranging from 3 to 17%. The MDL is defined as the lowest concentration of phenolic compounds which provide a greater than 99% confidence level when the procedure is used. MDLs of the nine phenolic compounds in the tested fish samples were within the range of 0.02–0.41 ng g^{-1} wet weight in fish; however, these MDLs could be decreased by increasing the sample size and injection volume during the GC analysis. This ensures a reliable determination at low levels compared to other reports [42–44].

The proposed analytical procedure was used to determine the level of phenols in fish collected from river near industrial area. As a positive fish sample containing nonylphenol (0.8 ng g^{-1}) and bisphenol A (1.8 ng g^{-1}), the ion chromatograms of TBDMS derivatized nonylphenol and bisphenol A are shown in Fig. 6. No significant interferences were observed in the ion chromatograms of TBDMS

Table 2 – Calibration equations, method detection limits (MDL) and recoveries of endocrine-disrupting phenols in fish tissue samples

Compound	Regression line			MDL (ng g^{-1})	Recovery ± R.S.D. (%)	
	Slope	Intercept	Correlation coefficient		1 ng g^{-1}	10 ng g^{-1}
1) 4-*tert*-Butylphenol	0.041	−0.009	0.995	0.20	102 ± 10	85 ± 10
(2) 4-*n*-Butylphenol	0.019	−0.004	0.995	0.02	78 ± 16	82 ± 10
(3) 4-Pentylphenol	0.089	−0.036	0.993	0.19	120 ± 8	108 ± 4
(4) 4-*n*-Hexylphenol	0.075	−0.047	0.993	0.17	78 ± 8	70 ± 3
(5) 4-*tert*-Octylphenol	0.124	−0.065	0.994	0.10	113 + 12	117 + 9
(6) 4-*n*-Heptylphenol	0.212	−0.138	0.994	0.14	103 ± 10	94 ± 10
(7) Nonylphenol	0.208	0.117	0.994	0.16	70 ± 13	75 ± 15
(8) 4-Octylphenol	0.167	−0.148	0.991	0.14	118 ± 9	100 ± 8
(9) Bisphenol A	0.225	−0.023	0.994	0.41	120 ± 17	105 ± 5

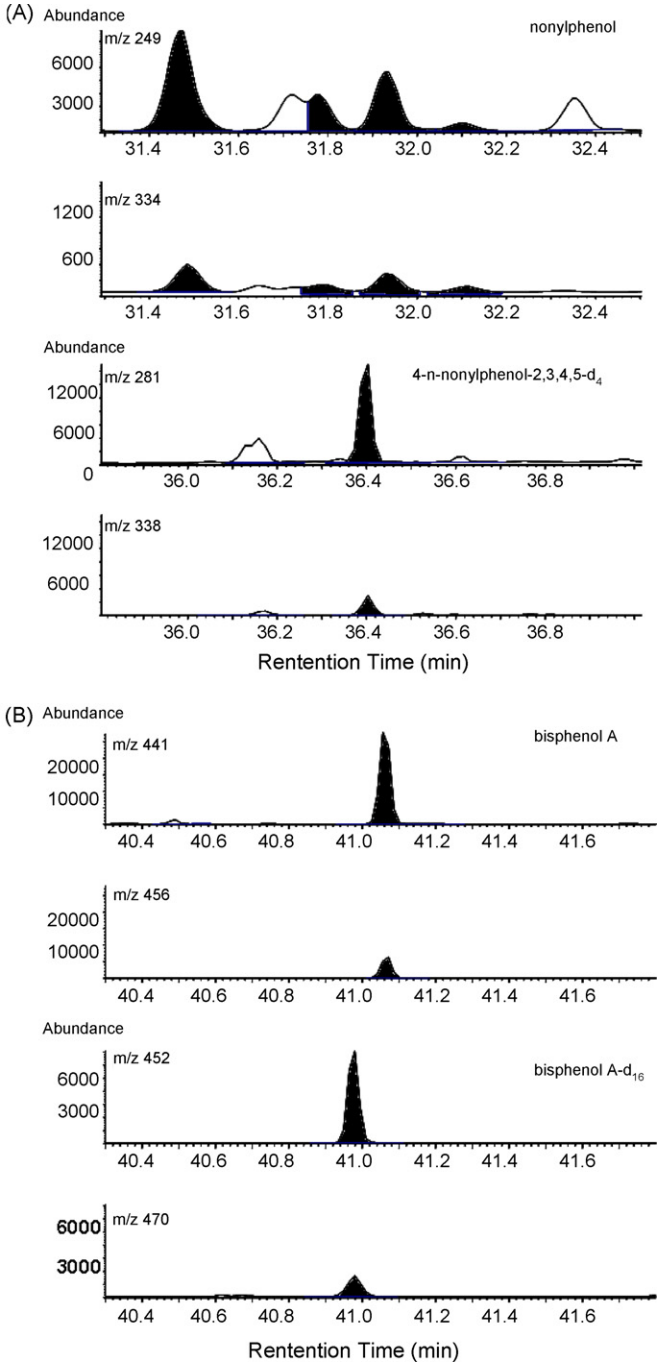

Fig. 6 – SIM chromatograms of TBDMS derivatized nonylphenol and its deuterium labeled internal standard (A), and bisphenol A and its deuterium labeled internal standard (B) in a positive fish sample.

derivatized bisphenol A and nonylphenol. Several peaks were observed in the chromatogram of nonylphenol-OTBDMS. The isomer patterns of nonylphenol-OTBDMS were used for the confirmation of nonylphenol. To quantify nonylphenol, four abundant peaks were selected for the integration of the peak areas. The detection and identification of other phenolic compounds was achieved on the basis of retention times and selected ion ratios with deuterium-labeled internal standards in the GC/MS-SIM.

4. Conclusion

The freezing-lipid filtration method enables efficient removal of lipids extracted from fish samples without any significant loss of phenols. The freezing-lipid filtration method, combined with dual SPE steps (HLB and Florisil-SPE), was simple and consumed little organic solvent as well as giving a satisfactory final result. Compared with other column clean-up methods for the removal of lipids extracted from biota samples, freezing-lipid filtration had a higher sample throughput and was easy to handle. Hence, the method can be used as a rapid screening tool for the determination of phenols in fish, on the basis of TBDMS derivatization and GC/MS analysis with deuterium-labeled phenols as internal standards. The spiking and recovery studies in fish tissue validated the analysis of phenols. Moreover, the analytical results, such as the recovery and method detection limit, were equivalent to or better than the official methods. Future work will explore the possibility of applying this method for the determination of phenolic compounds in the presence of high levels of lipids in other types of seafood.

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REFERENCES

[1] A.V. Krishnan, P. Stathis, S.F. Permuth, L. Tokes, D. Feldman, Endocrinology 132 (1993) 2279.
[2] S. Jobling, J.P. Sumpter, Aquat. Toxicol. 27 (1993) 361.
[3] J.E. Harries, D.A. Sheahan, S. Jobling, P. Matthiessen, J.P. Sumpter, T. Taylor, Environ. Toxicol. Chem. 16 (1997) 534.
[4] L.B. Christiansen, K.L. Pedersen, B. Korsgaard, P. Bjerregaard, Mar. Environ. Res. 46 (1998) 137.
[5] K. Sakurai, N. Sugaya, T. Nakagawa, T. Uchiyama, Y. Fujimoto, K. Takahashi, J. Health Sci. 51 (2005) 538.
[6] C.A. Staples, P.B. Dorn, G.M. Klecka, S.T. O'Block, L.R. Hariis, Chemosphere 36 (1998) 2149.
[7] S.N. Pedersen, L.B. Christiansen, K.L. Pedersen, B. Korsgaard, P. Bjerregaard, Sci. Total Environ. 233 (1999) 89.
[8] Nordic Council of Ministers, Copenhagen, TemaNord (1996) 580.
[9] S.N. Pedersen, C. Lindholst, J. Chromatogr. A 864 (1999) 17.
[10] J.R. Cejas, E. Almansa, J.E. Villamandos, P. Badia, A. Bolanos, A. Lorenzo, Aquaculture 216 (2003) 299.
[11] E. Almansa, M.J. Perez, J.R. Cejas, P. Badia, J.E. Villamandos, A. Lorenzo, Aquaculture 170 (1999) 323.
[12] G.H. Dodo, M.M. Knight, J. Chromatogr. A 859 (1999) 235.
[13] C. Orazio, J. Meadows, S. Kapila, C. Palmer, A.F. Yanders, Chemosphere 18 (1989) 69.
[14] G. Rimkus, M. Rummler, I. Nausch, J. Chromatogr. A 737 (1996) 9.
[15] M. Ribick, G. Dubay, J. Petty, D. Stalling, C. Schmitt, Environ. Sci. Technol. 16 (1982) 310.
[16] D. Stalling, R. Tindle, J. Johnson, J. AOAC 55 (1972) 32.
[17] J. Hong, Y. Eo, J. Rhee, T. Kim, K. Kim, J. Chromatogr. 639 (1993) 261.
[18] L.M. Smith, D.L. Stalling, J.L. Johnson, Anal. Chem. 56 (1984) 1830.
[19] L. Berdie, J.O. Grimait, J. Chromatogr. A 823 (1998) 373.

[20] L.G.M.Th. Tunistra, W.A. Traag, H.J. Keukens, J. Assoc. Off. Anal. Chem. 63 (1980) 952.
[21] R. Fuoco, S. Giannarelli, M. Onor, A. Ceccarini, V. Carli, Microchem. J. 79 (2005) 69.
[22] K. Kitamura, Y. Takazawa, S. Hashimoto, J.-W. Choi, H. Ito, M. Morita, Anal. Chim. Acta 512 (2004) 27.
[23] J. Hong, H.-Y. Kim, D. Kim, J. Seo, K. Kim, J. Chromatogr. A 1038 (2004) 27.
[24] J. Seo, H.-Y. Kim, B.C. Chung, J. Hong, J. Chromatogr. A 1067 (2005) 303.
[25] Y.G. Ahn, J. Seo, J.H. Shin, J. Khim, J. Hong, Anal. Chim. Acta 576 (2006) 31.
[26] M.J. Lopez de Alda, D. Barcelo, J. Chromatogr. A 892 (2000) 391.
[27] T. Tsuda, K. Suga, E. Kaneda, M. Ohsuga, J. Chromatogr. B 746 (2000) 305.
[28] B. Shao, H. Han, J. Hu, J. Zhao, G. Wu, Y. Xue, Y. Ma, S. Zhang, Anal. Chim. Acta 530 (2005) 245.
[29] M. Petrovic, A. Diaz, F. Ventura, D. Barcelo, Anal. Chem. 73 (2001) 5886.
[30] C.M. Lye, C.L.J. Frid, M.E. Gill, D.W. Cooper, D.M. Jones, Environ. Sci. Technol. 33 (1999) 1009.
[31] T.L. Keith, S.A. Snyder, C.G. Naylor, C.A. Staples, C. Summer, K. Kannan, J.P. Giesy, Environ. Sci. Technol. 35 (2001) 10.
[32] F. Ferrara, F. Fabietti, M. Delise, A.P. Bocca, E. Funari, Environ. Sci. Technol. 35 (2001) 3109.
[33] Y.C. Fiamegos, C.G. Nanos, G.A. Pilidis, C.D. Stalikas, J. Chromatogr. A 983 (2003) 215.
[34] A.T. Khan, S. Islam, A. Majee, T. Chattopadhyay, S. Ghosh, J. Mol. Catal. A 239 (2005) 158.
[35] K. Guenther, V. Heinke, B. Thiele, E. Kleist, H. Prast, T. Raecker, Environ. Sci. Technol. 36 (2002) 1676.
[36] S. Boitsov, S. Meier, J. Klungsøyr, A. Svardal, J. Chromatogr. A 1059 (2004) 131.
[37] EPA, Method 1613. Tetra through octa chlorinated dioxins and furans by isotopic dilution HRGC/HRMS. Washington DC, 1994.
[38] J. de Boer, Chemosphere 17 (1988) 1803.
[39] A. Covaci, S. Voorspoels, J. Chromatogr. B 827 (2005) 216.
[40] O. Pinho, I.M.P.L.V.O. Ferreira, M.A. Ferreira, Anal. Chem. 74 (2002) 5199.
[41] X.-Y. Xiao, D.V. McCalley, J. McEvoy, J. Chromatogr. A 923 (2001) 195.
[42] Japan Environmental Agency (1998) Environmental agency's initiatives to issues of exogenous endocrine disruptors. In Environmental Hormone strategic Project SPEED'98, Tokyo.
[43] I.S. Afonso, J.E.L. Rosales, M. de la, P. Aviles, B.A. Rattner, C.P. Rice, J. Chromatogr. A 1010 (2003) 25.
[44] S. Meier, J. Klungsøyr, S. Boitsov, T. Eide, A. Svardal, J. Chromatogr. A 1062 (2005) 255.