Rapid Determination of Chlorostyrenes in Fish by Freezing-Lipid Filtration, Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry

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An analytical method has been developed for measuring chlorostyrenes in fish tissue sample. Extraction of chlorostyrenes from fish tissue was carried out by ultrasonication using acetone/*n*-hexane (5:2, v/v) mixture. Most of the lipids in the extract were eliminated by freezing-lipid filtration, prior to solid-phase extraction (SPE) cleanup. During freezing-lipid filtration, about 90% of the lipids extracted from the fish samples were easily removed without any significant losses of chlorostyrenes. For purification, SPE using Florisil was used for the rapid and effective cleanup. Quantification was performed using gas chromatography-mass spectrometry in the selected ion monitoring mode. Spiking experiments were carried out to determine the recovery, precision, and limits of detection (LODs) of the method. The overall recovery was above 80% in the spiked fish tissue sample at 10 and 100 ng/g levels, respectively. The detection limits for chlorostyrenes were ranged from 0.05 to 0.1 ng/g. This developed method is demonstrated to give efficient recoveries and LODs for detecting chlorostyrenes spiked into fish tissue with high lipid content.

Key Words : Fish, Chlorostyrenes, Freezing-lipid filtration, SPE, GC-MS

Introduction

Although octachlorostyrene (OCS) is not commercially manufactured, it has been reported to be an incidental byproduct in the industrial process and in waste material burning at high temperature.[1] This compound is concerned as environmentally persistent pollutants due to its bioaccumulation in wild life and toxicity to aquatic organisms.[2-4] Moreover, OCS has been classified as one of endocrine disrupters, and monitored for various environmental samples such as water, soil, sediment and biological samples.[5] The measurement of OCS has been taken in various types of sample such as water, sediment, fish, bird's eggs, blood from workers, and humans with high consumption of fish in the factory.[6-16] In addition, monochlorostyrenes and dichlorostyrenes as degradation products of OCS have been detected in environment. The presence of these compounds could indicate that OCS was released to the environmental from industrial sources.[17] Furthermore, chlorostyrenes have been detected, in significant concentrations, in marine and freshwater habitats around the world. Continued development of analytical methodologies is needed for the accurate determination of chlorostyrenes in both environmental and biological samples.

Various analytical methods have been used to measure chlorostyrenes concentrations in sediment, fish and human blood[6-9] to determine not only the presence of them but also their concentrations in samples with precision and accuracy. Documented methods for quantitation of chlorostyrenes in biological samples utilized commonly capillary column gas chromatography (GC) with electron capture detector (ECD)[9] or combined with mass spectrometry (MS).[6,8,17] Prior to GC or GC/MS analysis, conventional approach to analyze chlorostyrenes in biological sample involves extraction followed by a multistep purification using various adsorbents. As extraction methods, Soxhlet extraction[6-8] and sonication extraction (SE)[18,19] have been widely used. Recently, Soxhlet extraction that requires overnight extraction and large amount of organic solvents is being replaced by rapid extraction methods such as pressurized liquid extraction (PLE),[20,21] microwave-assisted extraction (MAE),[22,23] supercritical fluid extraction (SFE)[24] and sonication extraction (SE). During the extraction of chlorostyrenes in biological tissue, large quantities of lipids may get inevitably co-extracted with targets due to their high solubility in organic solvents. In the case of GC or GC/MS analysis, lipid components tend to adsorb in GC system such as injection port and column, resulting in poor chromatographic performance. Several approaches including liquid-liquid partitioning,[25] gel permeation chromatography,[8] column chromatography[6,8] and multiple cleanup methods[26,27] have been reported to eliminate lipid interferences extracted from biological samples. However, most of these methods are time consuming and use large quantities of organic solvents to remove the fatty materials.

Recently, the freezing-lipid filtration method[18,28,29] has been successfully applied for elimination of lipids in biological samples. Large amount of lipids extracted from biological samples can be easily removed through the freezing-lipid in cold extract and the filtration. After freezing-lipid filtration, most of the remaining interference can be removed by a convenient solid-phase extraction (SPE) cartridge.

The purpose of this study is simultaneous determination of mono-, di-, and octa-chlorostyrenes in high lipid containing samples by the freezing-lipid filtration with simple SPE

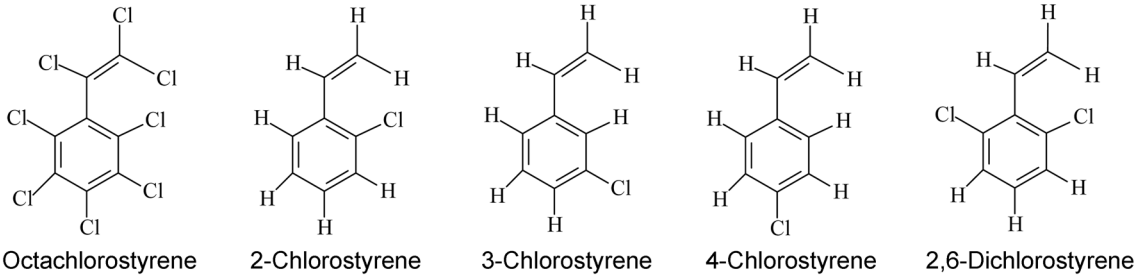

Figure 1. Chemical structures of chlorostyrenes investigated in this study.

cleanup and GC-MS. This developed method can be applied for the monitoring of chlorostyrenes regulated on fish in South Korea.

Experimental

Chemicals. Authentic chlorostyrenes were obtained from several companies: 2-chlorostyrene from Alfa Aesar (MA, USA). 3-chlorostyrene, 2,6-dichlorostyrene from Aldrich (Milwaukee, WI, USA) and 4-chlorostyrene, Octachlorostyrene from Wako Chemical (Tokyo, Japan). A stock standard mixture containing monochlorostyrene isomers, 2,6-dichlorostyrene and octachlorostyrene was prepared in acetone at a concentration of 1000 μg/mL, and stored at 4 ^{o}C. Working standard solution was prepared at the concentration of 0.01-0.4 μg/mL by volume, dilution with acetone.

Organic solvents (hexane, acetone and acetonitrile) were of pesticide residue analysis grade (J.T Baker, Phillipsburg, NJ, USA). Anhydrous sodium sulfate from Wako Chemical was used as the drying reagent. Prior to use, sodium sulfate was heated in muffle furnace at 550 ^{o}C for 12 h. All glassware were cleaned with laboratory detergent, sequentially rinsed with distilled water, acetone, and methanol, and finally baked in an oven at 300 ^{o}C. Distilled water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). For SPE, 5 g of Florisil were purchased from Supelco (Bellefonte, PA, USA). The chemical structures of the chlorostyrenes in this study are depicted in Figure 1.

For the precise analysis of chlorostyrenes, the stock solution should be freshly prepared every month, because some of the chlorostyrenes get degraded even when stored at −4 ^{o}C in a refrigerator. Moreover, the authentic standards should be stored in amber type bottles to protect photolytic degradation.

Equipment. Ultrasonic bath (Ultrason Selecta) was used for the extraction of chlorostyrenes from biota samples. The generator of ultrasonic bath has an output of 150 W and a frequency of 35 KHz. Rotary evaporator (EYELA, Tokyo, Japan) was used for the concentration of organic solvent.

Sample extraction and delipidation. Samples of 10 g together with 100 ng of [$^{13}C_6$]-HCB were ground in a blender far for 10 min at high speed. Extraction of chlorostyrenes from 10 g of biota samples was carried out by ultrasonic agitation with a mixed solvent of 70 mL of acetone/*n*-hexane (5:2, v/v) for 20 min. The extract was filtered with sodium sulfate, and then transferred into a 250 mL round flask. The extraction was repeated one more time. Extracted solvent was dried and redissolved in 50 mL of acetonitrile that has low solubility for lipids. Acetonitrile extract was stored in the freezer at −24 ^{o}C for 30 min to freeze lipids. Most of the lipids were precipitated as pale yellow, condensed lump on glassware surface. Cold extract at −24 ^{o}C was immediately filtered with filter paper to remove frozen lipids. The precipitated lipid on glassware surface was redissolved in 50 mL of acetonitrile to perform filtration again by same procedure. The filtered extract was concentrated to 1 mL by a rotary evaporator under nitrogen atmosphere to follow Florisil-SPE procedure.

Sample clean-up. Before sample application to SPE cartridge, the cartridge was cleaned with 12 mL of *n*-hexane and air dried by positive pressure for 1 min. Another 5 mL of *n*-hexane was used to condition the cartridge. After sample application, the Florisil-SPE cartridge was air-dried for 10 min. Desorption of the chlorostyrenes, which had been preconcentrated on the Florisil sorbent, was carried out using 15 mL of acetone/*n*-hexane (1:9, v/v) mixture at a flow of 1 mL/min and collected in a 50 mL round flask. The eluate

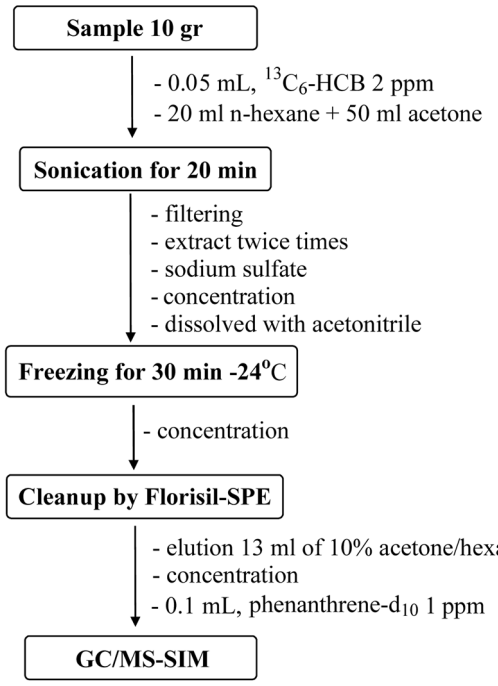

Figure 2. Analytical procedure of chlorostyrenes for fish tissue sample.

was then concentrated at 45 °C with a nitrogen stream until just the disappearance of the last drop of solution. Finally, 100 ng of phenathrene-d_{10} used as syringe internal standard was added to dried residue. The sample preparation scheme is represented in Figure 2.

GC-MS analysis. The sample analysis was carried out with an Agilent GC/MS (Palo Alto, CA, USA) equipped with DB-5MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA). Helium as carrier gas was used at a flow rate of 1.0 mL/min. A 1 μL sample was injected under split mode (split ratio 10:1). The temperatures of the GC injection port and the MS interface were set at 270 and 300 °C, respectively. The GC column temperature program was as follow: an initial temperature 100 °C, held for 3 min, and then ramp 10 °C/min to 280 °C held for 5 min.

The mass spectrometer was run in the electron ionization (EI) mode with electron energy at 70 eV. The instrumental parameters were set at the following values: 300 μA filament emission current and multiplier voltage of 2000 V. The manifold temperature was maintained at 200 °C. The mass spectrometer was operated with scan mode between 50 and 480 amu. For the monitoring and confirmation analysis, the selected ion monitoring (SIM) mode was used and the dwell time of each ion was set at 50 ms. To improve sensitivity, selected ions used in the SIM mode are divided into three groups, guiding by the individual chlorostyrene retention times. The selected ion groups in SIM mode are listed in Table 1. All chlorostyrenes were identified by retention time and specific ions, and quantified by the internal standard method.

Fortified recovery studies. Either 0.1 mL of 10 μg/mL spiking standard solution or 0.1 mL of a 1 μg/mL spiking standard solution was added to 10 g fish samples, which are equivalent to levels of 0.1, and 0.01 μg/mL in the tissue samples, respectively. Three replicates each at 0.1 μg/mL level and five replicates at 0.01 μg/mL level were prepared. All samples were extracted, purified and analyzed using GC-MS-SIM mode described above.

Results and Discussion

Extraction and clean-up. The sample was homogenized and extracted with a suitable solvent to remove the bulk of sample matrix and extract the chlorostyrenes compound residue into the solvent. Both the selection of solvent and extraction method can be critical for the satisfactory recovery of chlorostyrenes from the sample matrix. Methylene chloride or *n*-hexane has good dissolving capability for chlorostyrenes, but large amounts of lipids were co-extracted when a non-polar solvent was used. In case of using acetone and acetonitrile, fish tissue was aggregated during ultrasonic agitation, not enable to penetrate fish tissue. Thus, the mixture of acetone/*n*-hexane (5:2, v/v) was used as the ultrasonic extraction solvent for chlorostyrenes from the spiked fish tissue. Under these extraction conditions, the overall extraction yield of chlorostyrenes was above 95%, due to their good dissolving capability for chlorostyrene. Moreover, the extraction time can be greatly reduced using ultrasonic extraction instead of Soxhlet extraction.[30] The sonication method provides the equivalent recovery yield for chlorinated pollutants, compared to other extraction methods.

Table 1. Selected ion groups of chlorostyrenes in GC-MS-SIM analysis

Ion groups	Compounds	Time (min)	Quantitative ion (*m/z*)	Confirmation ion (*m/z*)
Group I	4-chlorostyrene	7.85	138	140
	2-chlorostyrene	7.91		
	3-chlorostyrene	7.98		
Group II	2,6-dichlorostyrene	10.56	172	174
Group III	$^{13}C_6$-HCB	12.48	290	292
Group IV	phenanthrene-d_{10}	15.81	188	–
Group V	octachlorostyrene	17.21	308	310

In general, complex mixtures of several types of lipids were co-extracted during the extraction of chlorostyrenes from biological sample. Triglycerides with unsaturated fatty acids and sterol esters are the major components in fish fats.[31,32] These compounds are less soluble in water but readily soluble in nonpolar solvents such as chloroform, methylene chloride and hexane. Thus, it is difficult to pretreat the sample extracts in order to selectively extract the interesting target as well as to remove the lipid-interferences from the extracts. Normally, several steps of cleanup including solvent partitioning and column cleanup were required to remove the lipid materials. These methods for removing lipids need a large amount of packing material, organic solvents, and time-consuming. Moreover, significant loss of volatile compounds could be occurred during a complex preparation process due to high volatility.

For the effective elimination of lipids extracted from biota sample, freezing-lipid filtration was applied for chlorostyrenes analysis. In our previous reports, this method has been successfully applied for the determination of chlorinated pesticides and polychlorinated dibenzo-*p*-dioxins/dibenzofurans in fish samples.[18,27] By using freezing-lipid filtration, approximately 90% of lipids in extraction solvent were eliminated without any significant loss of analytes in biota samples.

After freezing-lipid filtration, the extract has still presented about 10% of lipids, equivalent to about 100 mg in 10 g of fish tissue sample. Although a significant amount of lipids was eliminated by freezing-lipid filtration method, the amount of residual lipids was three-order higher than that of target analytes at sub-microgram level of the extract. Thus, the relatively high amount of remaining interferences such as lipids, fatty acids, and cholesterol should be eliminated by column chromatography. For further cleanup for the elimination of remaining interference after freezing lipid filtration, Florisil SPE cartridge was applied due to its simplicity, rapid and convenience. The elution patterns of chlorostyrenes on the Florisil SPE cartridge are shown in Figure 3. When acetone/*n*-hexane (1:9, v/v) was applied as elution solvent

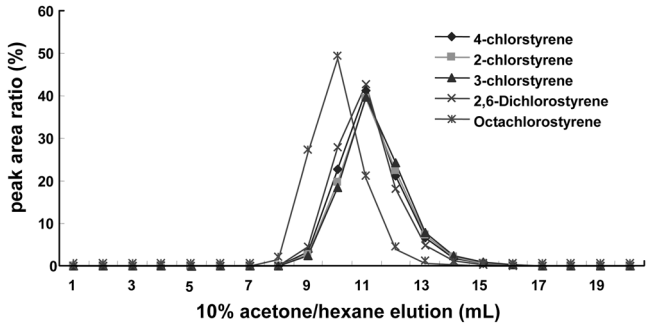

Figure 3. Elution patterns of chlorostyrenes on Florisil-SPE cartridge with acetone/*n*-hexane (1:9, v/v).

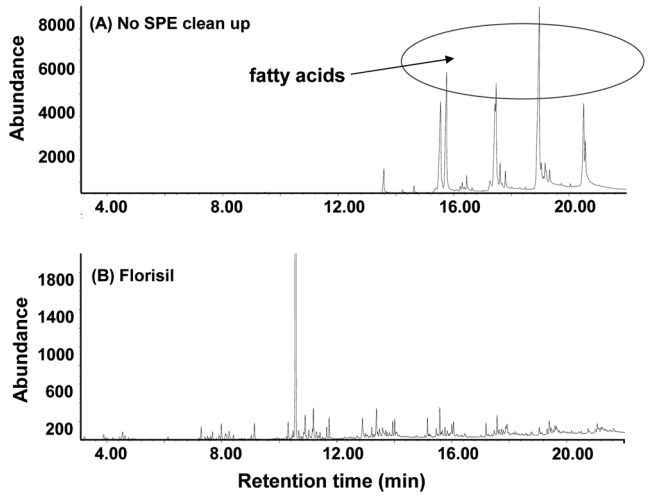

Figure 4. Total ion chromatograms of blank fish extracts after freezing-lipid filtration: no clean up (A) and Florisil-SPE (B).

on SPE cartridge, all analytes were completely eluted within in the range of 7 to 15 mL with 95-101% of recoveries.

Based on the elution patterns of chlorostyrenes on the Florisil SPE cartridge, the removal efficiency of interferences in extract after freezing-lipid filtration was attempted. Figure 4 shows total ion chromatogram (TIC) of the extract before (Figure 4-A) and after purified by Florisil- SPE cartridge (Figure 4-B) in GC-MS scan mode. Significant amount of several interferences such as fatty acids were still observed under without SPE cleanup, as shown in Figure 4-A. The main interference like fatty acids had retention time of 15 to 22 min, not enabling to obtain sufficient sensitivity for OCS in this range of retention time. On the other hand, when extract was purified by Florisil-SPE cartridge, significant amounts of fatty acids were efficiently eliminated, as shown in Figure 4-B. Although some of interferences were still detected due to incomplete removal by Florisil SPE cleanup, 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, Florisil-SPE and GC/MS-SIM.

Method application. Isotopic-labeled internal standard [$^{13}C_6$-HCB] and chlorostyrenes were spiked into the control fish sample, and then extracted, purified, and analyzed by the method developed. In order to evaluate the method

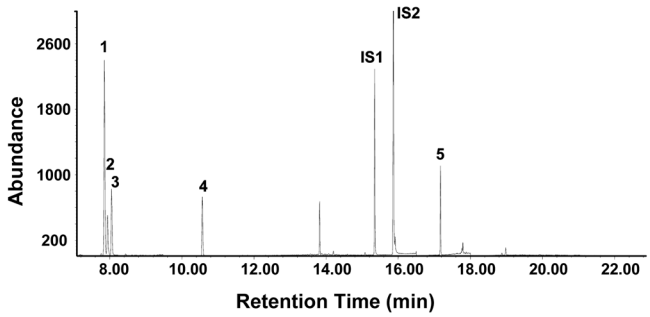

Figure 5. GC-MS-SIM chromatogram of chlorostyrenes in spiked fish sample at 10 ppb. Peak identities: 1. 4-chlorostyrene, 2. 2-chlorostyrene, 3. 3-chlorostyrene, 4. 2,6-dichlorostyrene, 5. octachlorostyrene, phenanthracene-d_{10} (IS1) and $^{13}C_6$-HCB (IS2).

Table 2. Recoveries and detection limits of chlorostyrenes in fish sample

No	Compounds	Recovery (%)		MDL (ng/g)
		10 ppb	100 ppb	
1	4-chlorostyrene	88.06 ± 8.49	82.43 ± 11.10	0.07
2	2-chlorostyrene	81.23 ± 7.26	79.99 ± 8.08	0.1
3	3-chlorostyrene	77.14 ± 7.86	85.67 ± 2.96	0.1
4	2,6-dichlorostyrene	84.59 ± 5.86	79.68 ± 5.52	0.05
5	octachlorostyrene	95.95 ± 1.39	95.48 ± 5.46	0.08

developed, 100 ng of internal standard and 100 ng of chlorostyrenes were spiked into a 10 g of blank fish tissue sample. Typical SIM chromatogram of the analysis of spiked fish tissue is shown in Figure 5. No significant interferences were observed in the total ion chromatogram. The ratios of the peak areas of chlorostyrenes and the corresponding internal standard were determined. The calibration curves were generated using a least-squares linear regression analysis ranging between 1 and 40 ng/g. The correlation coefficient for each chlorostyrenes was higher than 0.990. From this result, chlorostyrenes spiked in fish tissue at 10 ppb level were simultaneously detected with excellent sensitivity.

Chlorostyrenes-spiked fish tissue sample was repeatedly analyzed to determine the recovery, reproducibility and detection limits of the method. The mean and the relative standard deviations (R.S.D) of the recovery, calculated as the measured amount divided by the spiked amount are listed in Table 2. Recoveries of chlorostyrenes were between 79.68 and 95.48%, and the relative standard deviations are 2.96-11.10%, with an average of 84.65%. The detection limits of some chlorostyrenes were around 0.05-0.1 ppb at signal-to-noise ratio of 3 using two abundant ions in SIM mode. In view of their recoveries and removal of interference, freezing lipid filtration and Florisil-SPE cleanup is effective for the reliable confirmation and quantitation analysis of chlorostyrenes.

Conclusions

A rapid extraction, freezing-lipid filtration and GC-MS measurement method was developed used to measure chloro-

styrenes levels in a biota sample. The freezing-lipid filtration combined with Florsil-SPE cartridge enabled efficient removal of lipids extracted from fish sample without any significant loss of chlorostyrenes. Compared with other column cleanup methods for removing lipids extracted from biota sample, the freezing-lipid filtration showed higher throughput and was easy to handle, while generating equivalent analytical results. Hence, the method offered a rapid screening tool with high sensitivity and accuracy or the determination of chlorostyrenes in fish tissue, based on GC-MS-SIM and two internal standards. Spike and recovery studies in fish tissue validated the analysis of chlorostyrenes, particularly at fortifications most applicable to the fish tissue that were analyzed. Future work will examine further application of the method to analyze other type samples containing high level of lipids.

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References

1. Lahaniatis, E.; Clausen, E.; Fytianos, K.; Bieniek, D. *Neturwiss*. **1998**, *75*, 93.
2. Selden, A.; Nygren, Y.; Westberg, H.; Bodin, L. *Occp. Environ. Med*. **1997**, *54*, 613.
3. Lommel, A.; Kruse, H.; Muller, E.; Wassermann, O. *Arch. Environ. Contam. Toxicol*. **1992**, *22*, 14.
4. Tarkpea, M.; Hagen, I.; Carlberg, G.; Kolsaker, P.; Storflor, H. *Bull. Enviorn. Contam. Toxicol*. **1985**, *35*, 525.
5. Database of Octachlorostyrene, Compilation prepared by Environmental Protection Agency (EPA), http://www.epa.gov/pbt/pubs/octaaction.htm#ref.
6. Chu, S.; Covaci, A.; Voorspeols, S.; Schepens, P. *J. Environ. Monit*. **2003**, *5*, 619.
7. Kaminsky, R.; Hltes, R. *Environ. Sci. Technol*. **1984**, *18*, 275.
8. Bester, K.; Biselli, S.; Ellerichmann, T.; Huhnerfuss, H.; Moller, K.; Rimkus, G.; Wolf, M. *Chemosphere* **1998**, *37*, 2459.
9. Boer, J.; Valk, F.; Kerkhoff, M.; Hagel, P. *Environ. Sci. Technol*. **1994**, *28*, 2242.
10. Kuehl, D.; Haebler, R. *Arch. Environ. Contam. Toxicol*. **1995**, *28*, 494.
11. Samdau, C.; Meerts, I.; Letecher, R.; Mcalees, A.; Chittim, B.; Brouwer, A.; Norstorm, R. *Environ. Sci. Technol*. **2000**, *34*, 3871.
12. Boer, J.; Zande, T.; Pieters, H.; Ariese, F.; Schipper, C.; Brummelen, T.; Vethaak, D. *J. Environ. Monit*. **2001**, *3*, 386.
13. Selden, A.; Westberg, H. *Arch. Environ. Health* **1999**, *54*, 248.
14. Kuehl, D.; Kopperman, H.; Veith, G.; Glass, G. *Bull. Environ. Contam. Toxicol*. **1976**, *16*, 127.
15. Green, N.; Knutzen, J. *Mar. Pollut. Bull*. **2003**, *46*, 362.
16. Fisk, A.; Holst, M.; Hobson, K.; Duffe, J.; Moisey, J.; Norstorm, R. *Arch. Environ. Contam. Toxicol*. **2002**, *42*, 118.
17. Coelhan, M.; Reil, I.; Rimkus, G.; Parlar, H. *Environ. Sci. Technol*. **2000**, *34*, 4695.
18. Hong, J.; Kim, H.; Kim, D.; Seo, J.; Kim, K. *J. Chromatogr. A* **2004**, *1038*, 27.
19. Castro, J.; Sanchez-Brunete, C.; Tadeo, J. *J. Chromatogr. A* **2001**, *918*, 371.
20. Richter, B.; Jones, B.; Ezzell, J.; Porter, N.; Avdalovic, N.; Pohl, C. *Anal. Chem*. **1996**, *68*, 1033.
21. Ramos, L.; Kristenson, E.; Brinkman, U. *J. Chromatogr. A* **2002**, *975*, 3.
22. Eskilsson, C.; Bjorklund, E. *J. Chromatogr. A* **2002**, *902*, 227.
23. Zuloaga, O.; Etxebarria, N.; Fernandez, L.; Madariaga, J. *Talanta* **1999**, *50*, 345.
24. Turner, C.; Eskilsson, C.; Bjorklund, E. *J. Chromatogr. A* **2002**, *947*, 1.
25. Kitamura, K.; Takazawa, Y.; Hashimoto, S.; Choi, J.; Ito, H.; Morita, M. *Anal. Chim. Acta* **2004**, *512*, 27.
26. Smith, L.; Stalling, D.; Johnson, J. *Anal. Chem*. **1984**, *56*, 1830.
27. Ryan, J.; Pilon, J.; Conacher, H.; Firestone, P. *J. Assoc. Off. Anal. Chem*. **1983**, *66*, 700.
28. Ahn, Y.; Seo, J.; Shin, J.; Khim, J.; Hong, J. *Anal. Chim. Acta* **2006**, *576*, 31.
29. Seo, J.; Kim, H.; Chung, B.; Hong, J. *J. Chromatogr. A* **2005**, *1067*, 303.
30. Tor, A.; Aydin, M.; Ozcan, S. *Anal. Chim. Acta* **2006**, *559*, 173.
31. Cejas, J.; Almansa, E.; Villamandos, J.; Badia, P.; Boalnos, A.; Lorenzo, A. *Aquaculture* **2003**, *216*, 299.
32. Almansa, E.; Perez, M.; Cejas, J.; Badia, P.; Villamandos, J.; Lorenzo, A. *Aquaculture* **1999**, *170*, 323.