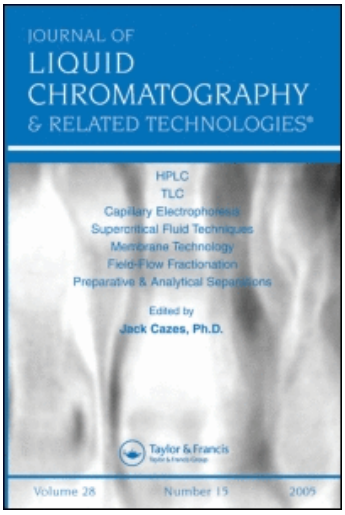

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Enrichment of Trace Chlorinated Species in a Complex Matrix of Fatty Acids Using HPLC in Conjunction with Gas Chromatography-Halogen Specific Detection

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Enrichment of Trace Chlorinated Species in a Complex Matrix of Fatty Acids Using HPLC in Conjunction with Gas Chromatography-Halogen Specific Detection

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ABSTRACT

Due to the similarity in chemical and physicochemical properties between analytes (chlorinated fatty acids) and matrix compounds (nonchlorinated fatty acids), conventional cleanup procedures were not successful in analysis of chlorinated fatty acids in extracts of freshwater fish. A new approach was devised of utilizing reversed-phase high performance liquid chromatography (HPLC) for its high separation power, and the halogen

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specific detector (XSD) for its high selectivity for organochlorine. The bulk of nonchlorinated matrix was removed by HPLC fractionation, and target chlorinated analytes were selectively enriched. The enrichment effect was assessed by a universal detector, a flame ionization detector (FID). Methyl esters of chlorinated fatty acids were completely nondetectable prior to HPLC fractionation, but were present as moderate or small, yet discernible, peaks after the HPLC enrichment. This enrichment method is efficient with good selectivity, reproducibility, and predictability.

Key Words: Fish extracts; Lipids; Chlorinated fatty acids; HPLC; Enrichment; Halogen-specific detection; Bleached kraft pulp mill effluent.

INTRODUCTION

Structural analysis of traces of chlorinated fatty acids in biota often requires prior enrichment or cleanup. Analysis of chlorinated pesticides, sometimes referred to as organochlorines (OCs), and polychlorinated biphenyls (PCBs) can be facilitated by a simple cleanup procedure, such as the sulfuric acid treatment or gel permeation chromatography (GPC), followed by silica gel chromatography. These OCs and PCBs account for only a small fraction of total extractable organochlorine (EOCl) measured in biota.[1] Behaving very differently from OCs and PCBs, the dominant EOCl components are destroyed by the sulfuric acid treatment and co-eluted with lipids in GPC. Due to difficulty in cleanup or enrichment for the bulk of the EOCl, their chemical nature had been a mystery until significant work was carried out by Wesén and coworkers in 1992.[2] These researchers analyzed an extract of a high EOCl load (1200 ppm) from eels caught in a fiord receiving untreated effluent from a bleached sulfite pulp mill using chlorine bleaching. After transesterification, or hydrolysis followed by methylation to produce fatty acid methyl esters (FAMEs), methyl 9,10-dichlorooctadecanoate was tentatively identified using a gas chromatograph (GC) equipped with an electrolytic conductivity detector (ELCD). Using this detector, which is highly specific to halogenated species, chlorinated fatty acids were estimated to account for 90% of the EOCl in the eel lipid.[2]

Mass spectrometry (MS) coupled with chromatography is commonly used for identification and confirmation of unknowns. Mass spectrometry can offer much structural information needed for identification and confirmation, but it is less selective and less sensitive than those detectors designed specifically for organohalogen such as ELCD[2] and halogen specific detector (XSD).[3] In order to obtain meaningful mass spectra, Wesén et al. enriched chlorinated

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FAMEs by cold trapping of the GC eluate.[4] Methyl 9,10-dichlorooctadecanoate was consequently confirmed by GC-MS, with an additional component, methyl 9,10,12,13-tetrachlorooctadecanoate being identified in this enriched specimen. Later, they adopted a different strategy for enriching chlorinated FAMEs, in which transesterified fish extracts were treated consecutively by silver nitrate in aqueous ethanol and urea in organic solvents, and sometimes additionally treated by silica gel thin layer chromatography.[5] Such an enrichment resulted in a 30-fold increase in the concentration of chlorinated FAMEs. Shortly thereafter, Milley et al. reported a reinforced enrichment, in which transesterified extracts of lobster digestive glands were enriched for chlorinated FAMEs, first by the urea treatment and then further, by gel permeation chromatographic fractionation (Sephadex LH-20).[6] One of the GPC fractions contained a high concentration of methyl dichlorotetradecanoate and, thus, permitted its identification and confirmation by GC-negative chemical ionization mass spectrometry (GC-NCIMS).

High performance liquid chromatography (HPLC) has been widely used for analytical separations by virtue of its high resolution capability. Though it is logical to use HPLC for enrichment of chlorinated fatty acids present in a complex matrix, to our knowledge, no such work has been reported. The difficulty in this application is associated with LC detectors, since none of them are specific for chlorine. As a result, the elution profile of traces of chlorinated compounds cannot be obtained from LC detectors. In this study, the high resolving power of reversed-phase HPLC was exploited for selective enrichment of chlorinated FAMEs in the matrix of non-chlorinated FAMEs. Inability of LC detectors for detecting traces of chlorinated FAMEs was circumvented by using XSD; a GC detector highly selective for halogen-containing compounds. The samples for this study were obtained by transesterification of filet extracts from fish sampled in a Canadian river downstream from a bleached kraft pulp mill and in a nearby reference river.[7,8] The transesterified extracts, composed mainly of FAMEs, contained EOCl at levels ranging from 15 to 103 μg Cl/g FAMEs.

EXPERIMENTAL

Sample Preparation

White sucker (*Catostomus commersoni*) were collected in a river downstream from a bleached kraft mill and in a pristine river, located in the Moose River basin in northern Ontario.[9] Filets from the fish samples were ground, freeze-dried, and extracted with hexane : acetone (1 : 1) in an accelerated solvent extractor (ASE 200, Dionex) at 55°C for 10 min, and then further,

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at 100°C for 5 min. The extracts were washed with Nanopure water at pH 3.0 (acidified with H_2SO_4), dried over anhydrous Na_2SO_4, filtered, and concentrated in a stream of N_2 to dryness.

Using cyclohexane as an eluent, an aliquot of filet extract was then eluted through a pre-flushed 2.5×60 cm glass column packed with Bio-Beads SX3 and separated into two fractions: high molecular weight (HMW) and low molecular weight (LMW). The cutoff for collecting these two fractions was about 230 Dalton, estimated from the elution profile of a mixture of triolein and biphenyl. The collected fractions were washed with Nanopure water, dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness.

About 0.6 g of the HMW fraction of a filet extract was dissolved in 1–2 mL of toluene, and 5 mL of methanol containing 2% (v/v) of H_2SO_4 were added. The mixture solution was left overnight in a capped tube at 50°C. Water (10 mL) was added, and then the suspension was extracted with hexane (3×10 mL). The combined hexane layers were washed with water (10 mL) containing $KHCO_3$ (2%) and then with water (10 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated in a stream of N_2 to dryness. The EOCl concentration in the resultant transesterified extract was measured by neutron activation analysis (NAA).

Fractionation by High Performance Liquid Chromatography

A transesterified fish extract, or a sample that had been pre-enriched by a urea method,[8] was fractionated using a Waters HPLC system consisting of a Model 600 multisolvent delivery system equipped with a U6K injector and controlled by a Model 600E system controller, a Model 991 programmable photodiode array detector (PAD), a Model 990 plotter, and a microcomputer to control the PAD, interface with other modules and process chromatograms. The column was Waters Spherisorb® ODS2 analytical column of 4.6 (I.D.) × 250 mm (L) with particle size 5 μm and pore size 80 Å. The flow rate was 0.8 mL/min and the eluate monitored at 215 nm. A gradient elution, shown in Table 1, was optimized for enriching the major components of fish EOCl in the specimens, after trial and error using guidelines set by Snyder et al.[10] Repeated runs were carried out to collect sufficient material for further work. For transesterified extracts that had been stored for more than a week, re-methylation with diazomethane was conducted prior to HPLC fractionation, to ensure that all fatty acids were in the ester form. Each fraction pooled from repeated runs was concentrated to dryness in a stream of N_2, re-methylated with diazomethane, and subjected to GC-XSD and flame ionization detector (FID) analysis.

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Table 1. Gradient elution.[a]

Time (min)	Water (%)	Methanol (%)	Acetonitrile (%)	Cyclohexane : isopropanol (1 : 1) (%)
0	10	80	10	0
18	0	89	11	0
26	0	89	11	0
30	0	60	20	20
40	0	89	11	0
45	10	80	10	0

[a]Linear curve was used for changing the composition of the mobile phase.

Gas Chromatography Analysis

Gas chromatography was performed in an HP 6890 GC equipped with an FID and an OI 5360 XSD. Each of the detectors was connected to a separate column (30 m × 0.25 mm × 0.25 μm), either HP-5MS or DB-WAX. The FID was operated at 310°C with flow rates being 40 mL/min for H_2, 45 mL/min for N_2 (makeup), and 450 mL/min for air; and the XSD at 1100°C with an air flow of 40 mL/min. The two parallel columns shared an injector, operated at 270°C in the splitless mode. Helium at 1.2 mL/min was used as the carrier gas. The oven temperature was maintained at 80°C for 1 min, and increased at 20°C/min to 160°C, then at 4°C/min to 284°C, and finally, at 20°C/min to 310°C. The corresponding retention times in the FID and XSD chromatograms were correlated by running a mixture of dichloro FAME standards.

RESULTS AND DISCUSSION

Correlation Between Chromatograms Recorded by the Flame Ionization Detector and Halogen Specific Detector Chromatograms

To have a maximum throughput for trace analysis, the injected sample was run through two parallel columns and recorded by XSD and FID fitted at the discharge ends of the columns. Since the elution conditions in the two parallel columns are not exactly the same, retention times in the resulting two chromatograms would not be the same in numeric values. Nevertheless, the retention times of analytes in the two columns can be paired off using a linear regression equation based on standards. It was found that, retention

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times of dichloro FAME standards in the dual-column runs have excellent linear relationships, with the correlation coefficient in excess of 0.9999. Figure 1 shows the XSD and FID chromatograms of a mixture of dichloro FAME standards after the retention time axis in the FID chromatograms was scaled according to the regression equation based on the retention times of corresponding peaks in the two chromatograms. As we see in Fig. 1, the corresponding peaks in the two chromatograms are almost perfectly aligned. In comparative analysis of the XSD and FID chromatograms of a sample, the standard mixture for establishing a linear relationship was run in the same sequence as was the sample, to minimize the fluctuation in the performance of GC elution and detectors between sequences, particularly when there was an intervening period in which the system was turned off. The correspondence of peaks of chlorinated species in the XSD and FID chromatograms of an HPLC fraction or enriched sample was determined by alignment after proper scaling of the retention time axis in one of the chromatograms. If paired peaks in the two chromatograms were truly derived from the same chlorinated FAME, the height and area ratios of the two peaks were expected to be about the same as those obtained from the corresponding standard run in the same sequence.

The same technique can be used for correlating retention times of analytes in chromatograms from runs for which operational conditions may vary.

Enrichment of Major Components of Chlorinated Fatty Acid Methyl Esters by Reversed-Phase High Performance Liquid Chromatography

Figure 2 is an HPLC chromatogram recorded by UV at 210 nm. As shown in the chromatogram, chlorinated FAME standards were well separated by HPLC. It appears that chlorinated FAMEs with shorter hydrocarbon chains were eluted earlier than those having longer chains. This elution order was anticipated, since in the reversed-phase HPLC, the interaction of eluted species with the stationary phase occurs mainly on the hydrophobic portion of the eluted molecules.

Specimens derived from transesterification of filet extracts after GPC removal of LMW (<∼300 Daltons) materials, were thus eluted with HPLC using the established chromatographic conditions described in the Experimental section. The fish were sampled in two different years from three rivers: the Mattagami River (downstream from a bleached kraft pulp mill), the Kapuskasing River (downstream from a thermomechanical pulp mill), and the Groundhog River (a pristine river). It was found that the HPLC chromatograms of transesterified extracts were similar, regardless of the sampling location, sampling year, and fish gender. Figure 3 is a typical HPLC

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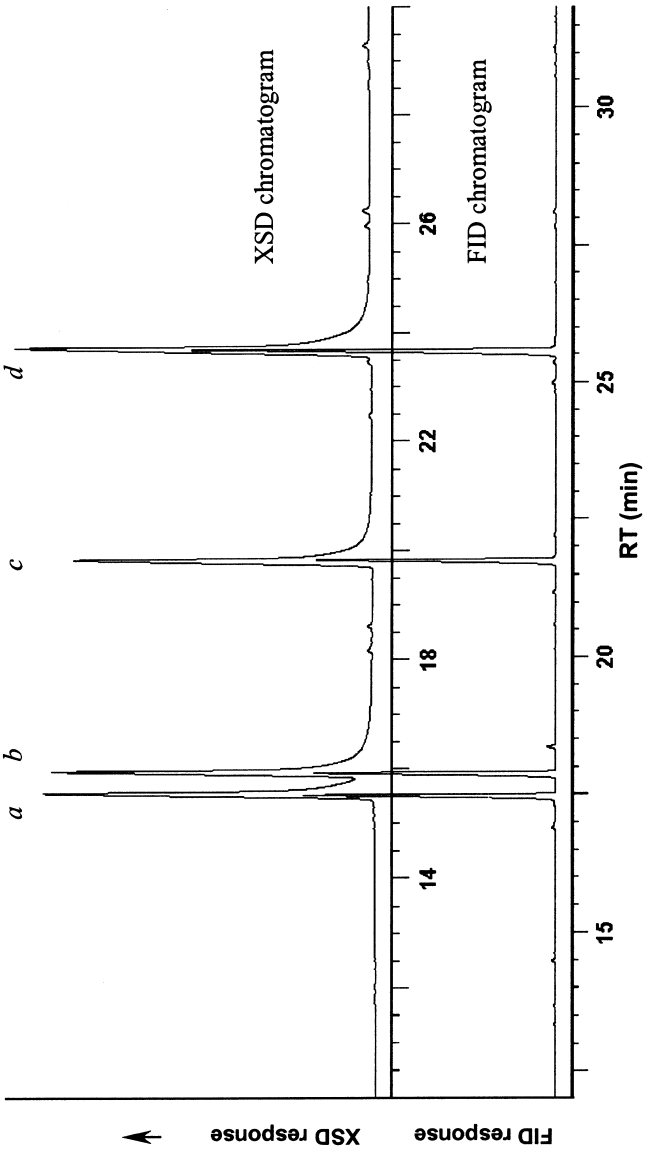

Figure 1. Correlation of the FID and XSD chromatograms (two parallel columns) of a mixture of standards. The retention time (RT) axis in FID chromatogram was scaled using the equation: RT in FID = 1.0192 × RT in XSD + 1.8182. *a*—Methyl *threo*-5,6-dichlorotetradecanoate, *b*—methyl *threo*-9,10-dichlorotetradecanoate, *c*—methyl *threo*-9,10-dichlorohexadecanoate, and *d*—methyl *threo*-9,10-dichlorooctadecanoate.

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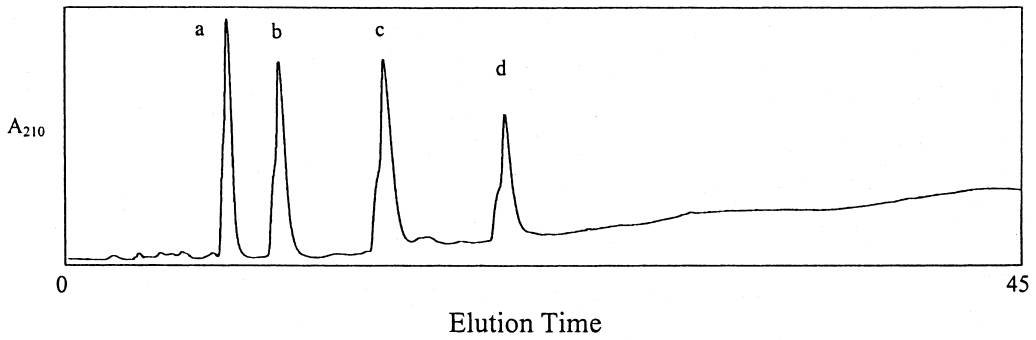

Figure 2. Separation of a mixture of methyl esters by reversed-phase high performance liquid chromatography. (a) *Threo*-5,6-dichlorododecanoate, (b) *threo*-9,10-dichlorotetradecanoate, (c) *threo*-9,10-dichlorohexadecanoate, and (d) *threo*-9,10-dichlorooctadecanoate.

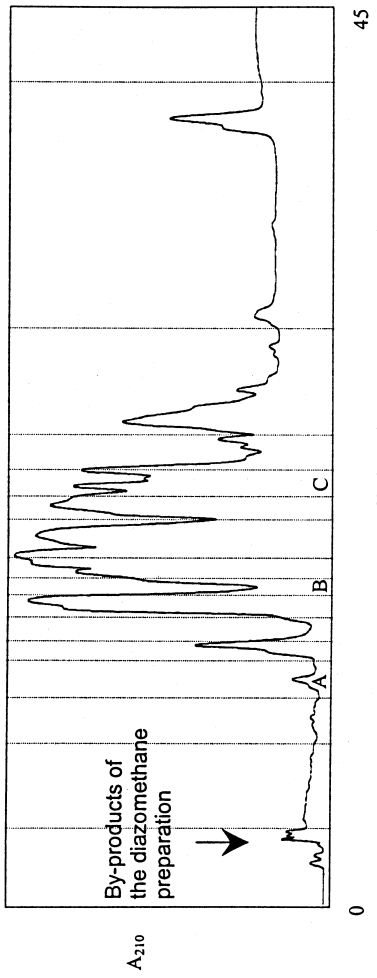

Figure 3. Reversed-phase HPLC chromatogram of a transesterified fish extract. Fractions A, B, and C contain analytes of interest (*vide* Fig. 5).

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chromatogram of these samples. Although they were too complex to be resolved by the HPLC elution, components of the transesterified extract spread over a range of at least ten bandwidths of dichloro FAMEs in the HPLC elution profile (Fig. 3). The vertical dashed lines shown in Fig. 3 indicate demarcation points for fractionation. The UV response in the elution profile arose from matrix components; concentrations of chlorinated components in the specimen were too low to see in the elution profile.

The fractions that contained major chlorinated FAMEs were examined by GC–XSD analysis. In practice, GC chromatograms were obtained using two parallel columns with simultaneous detection by XSD and FID. The XSD revealed traces of chlorinated FAMEs while the FID recorded the elution profile according to concentrations of eluates, which were dominated by non-chlorinated FAMEs. Locations of chlorinated FAMEs in the FID chromatogram were determined by alignment with the XSD chromatogram after proper scaling. The scaling was done using the linear regression equation of retention times in the FID and XSD chromatograms of standards, as described earlier. Comparing the XSD and FID chromatograms of HPLC fractions helped assess the HPLC elution profile of chlorinated analytes against nonchlorinated matrix materials, and determine the demarcation points of fractionation accordingly. The elution conditions of HPLC could be adjusted to avoid co-eluting chlorinated analytes with high concentrations of matrix compounds. This was shown by a gradient elution developed for the study samples, by which the target analytes were all present in the valleys of the HPLC chromatogram of matrix materials (Fig. 3). Thus, the bulk of the matrix could be removed by HPLC fractionation and marked enrichment achieved.

The effectiveness of the HPLC fractionation on enrichment of chlorinated analytes can be demonstrated by GC–XSD and GC–FID chromatograms of the extracts before and after HPLC fractionation. The specimen, derived from extracts of male fish collected in the pristine river, displayed a prominent peak in the XSD chromatogram [Fig. 4(A)]. This peak was completely invisible in the FID chromatogram [Fig. 4(B)]. In HPLC fractionation, the chlorinated component responsible for this XSD peak was mostly collected in a fraction just before fraction A labelled in Fig. 3. A majority of the matrix compounds was, thus, excluded from this HPLC fraction. The XSD and FID chromatograms of this fraction are shown in Fig. 4(C)(D), respectively. As we see, the chlorinated component now appears as a significant peak in the FID chromatogram. This compound was later identified as an isomer of methyl dichlorotetradecanoate.[11]

In transesterified extracts of the fish collected downstream of the bleached kraft mill, three major chlorinated FAMEs, methyl *threo*-5,6-dichlorotetradecanoate, 6,7-dichlorohexadecanoate, and 9,10-dichlorooctadecanoate, were identified by GC–XSD analysis.[12] The chromatograms of the specimen

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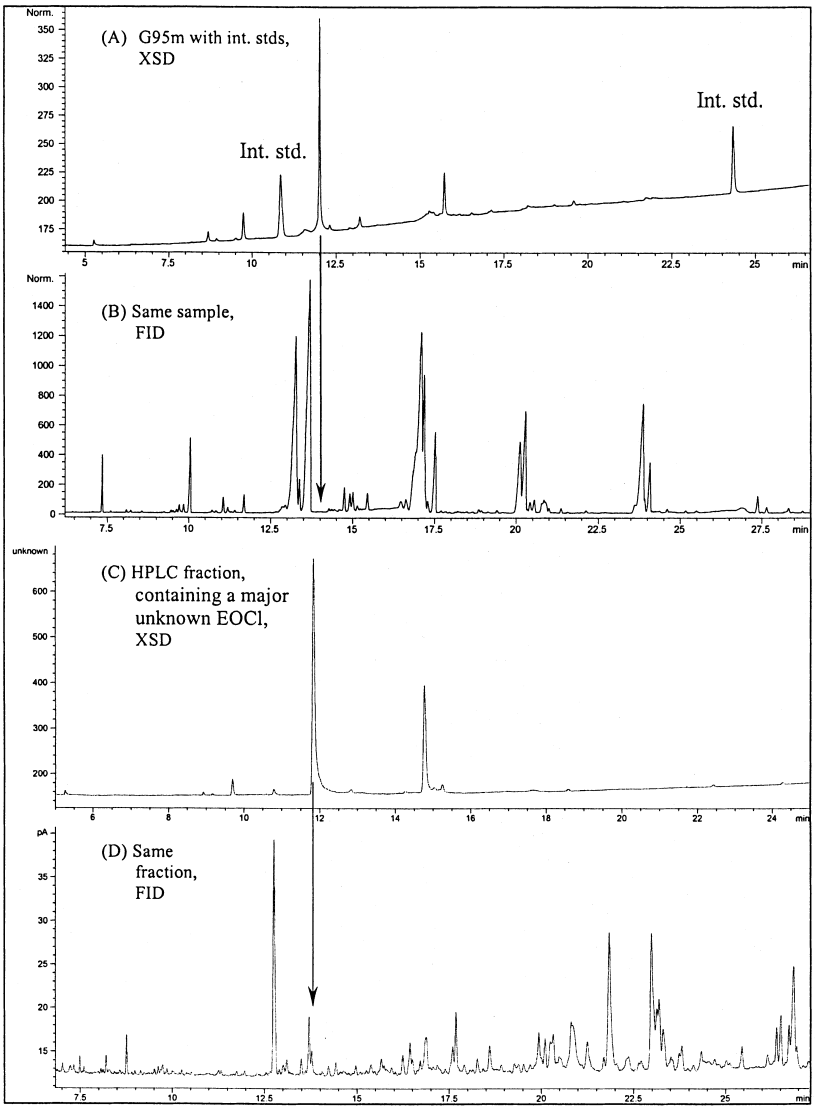

Figure 4. Enrichment effect of HPLC fractionation for a target compound in a transesterified extract (43 μg Cl/g) prepared from filets of the male fish sampled in the Groundhog River in 1995 (G95m). Int. stds: methyl esters of *threo*-5,6-dichlorododecanoic acid and *threo*-10,11-dichlorononadecanoic acid. Column: HP-5.

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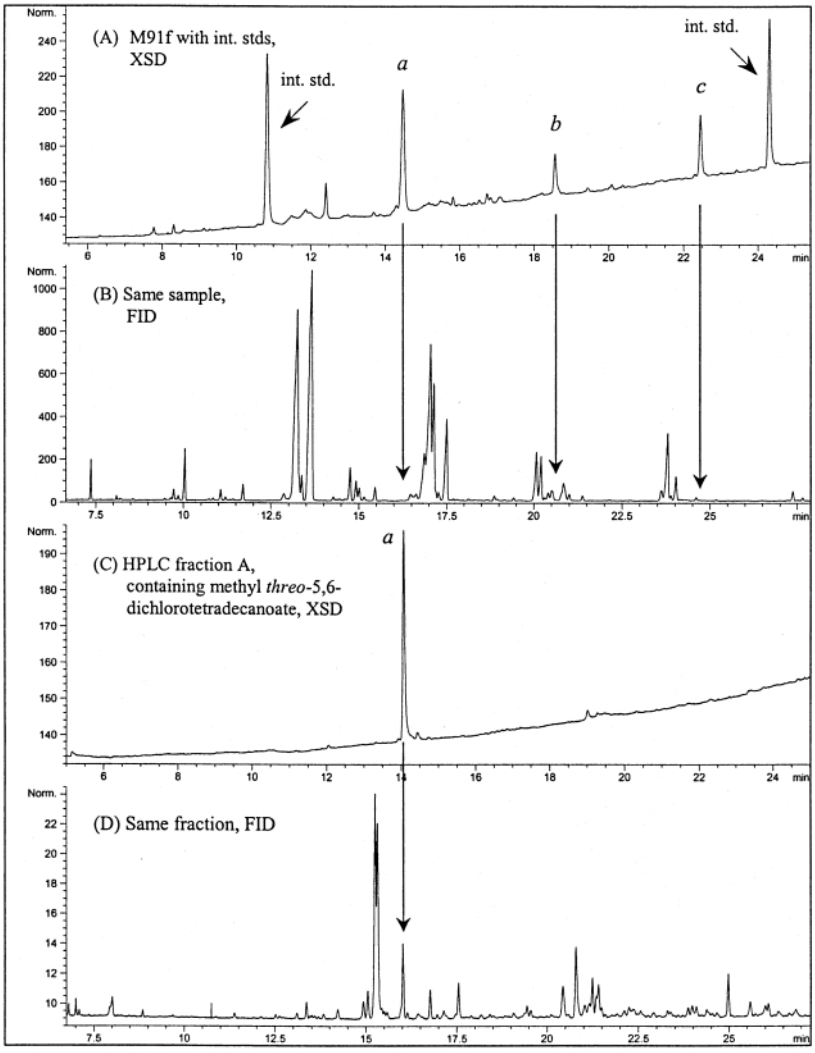

Figure 5. Enrichment effect of HPLC fractionation for target analytes *a*, *b* and *c* in a transesterified extract (103 µg Cl/g) prepared from filets of the female fish sampled downstream from the bleached kraft mill in 1991 (M91f). Int. stds: the same as in Fig. 4.

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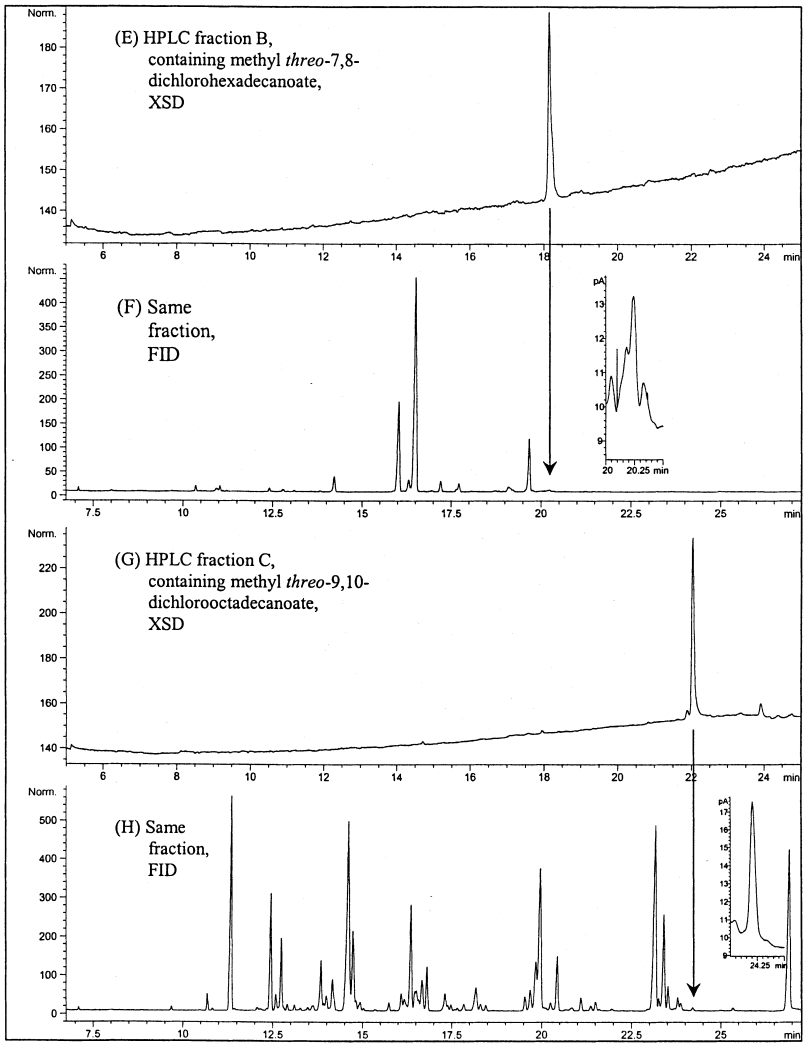

Figure 5. Continued.

prior to HPLC fractionation are shown in Fig. 5(A)(B), where (A) is the chromatogram recorded by XSD and (B) by FID. Figure 5(A) shows that these three compounds, labelled as *a*, *b*, and *c*, were major chlorinated analytes in the specimen. However, their concentrations were so low that they were not traceable at all by FID [Fig. 5(B)]. In this FID chromatogram, chlorinated

analytes were overwhelmed by the matrix compounds. Under the HPLC conditions adopted in this study, these chlorinated FAMEs were collected in fractions labelled as A, B, and C in Fig. 3. As we see in Fig. 3, co-elution of these chlorinated FAMEs with predominant matrix components was avoided. Enriched in fraction A, methyl *threo*-5,6-dichlorotetradecanoate shown as peak *a* in Fig. 5(A)(C), was displayed as a significant peak in the FID chromatogram [Fig. 5(D)]. Though its concentration was still low in the fraction B, methyl *threo*-7,8-dichlorohexadecanoate [peak *b* in Fig. 5(A)(E)] was now barely detectable by FID [Fig. 5(F)]. There are about four peaks present around the position, which is aligned with the XSD peak. Though it is beyond the precision of the RT correlation to determine which of these so closely eluted peaks is the one corresponding to the XSD peak, only pairing of the highest peak with the XSD peak gives the peak height and area ratios that matches those of the standard. The concentration of methyl *threo*-9,10-dichlorooctadecanoate [peak *c* in Fig. 5(A)(G)] was similarly low in fraction C, yet it was readily discerned by FID and there were less co-eluted interferences [Fig. 5(H)]. The FID chromatograms of these fractions demonstrate an additional advantage of HPLC fractionation; though the enrichment for methyl *threo*-7,8-dichlorohexadecanoate and 9,10-dichlorooctadecanoate was moderate, major matrix compounds in the HPLC fractions did not interfere with these analytes in GC analysis.

The last example to illustrate HPLC fractionation is a transesterified extract, which showed no peaks even in the GC-XSD chromatogram [Fig. 6(A)]. Reversed-phase HPLC fractionation produced several fractions with levels of chlorinated FAMEs that exceeded the detection limit of the XSD. Figure 6(B) shows the GC-XSD chromatogram of a specimen derived from combining the four HPLC fractions that contained most of chlorinated FAMEs in the original sample.

The degree of enrichment of a particular chlorinated FAME by HPLC fractionation can be estimated by simple algebra. Suppose that f% of a chlorinated FAME is collected in an HPLC fraction, and that the mass of this fraction is y% of the total mass of the sample. Then, the concentration enhancement of this chlorinated FAME will be f/y fold. It was estimated in the experiment that the enrichment for the major chlorinated component in the reference sample was about 150-fold. The HPLC fractionation of a transesterified extract derived from the exposed fish resulted in an enhancement of 90-fold for methyl *threo*-5,6-dichlorotetradecanoate, 15-fold for methyl *threo*-7,8-dichlorohexadecanoate, and 7-fold for methyl *threo*-9,10-dichlorooctadecanoate. Another feature of the enrichment by HPLC fractionation is that peaks of matrix compounds are often spread out in GC chromatograms and predominant matrix compounds are often well separated from chlorinated FAMEs. This is because elution mechanisms of HPLC and GC are quite

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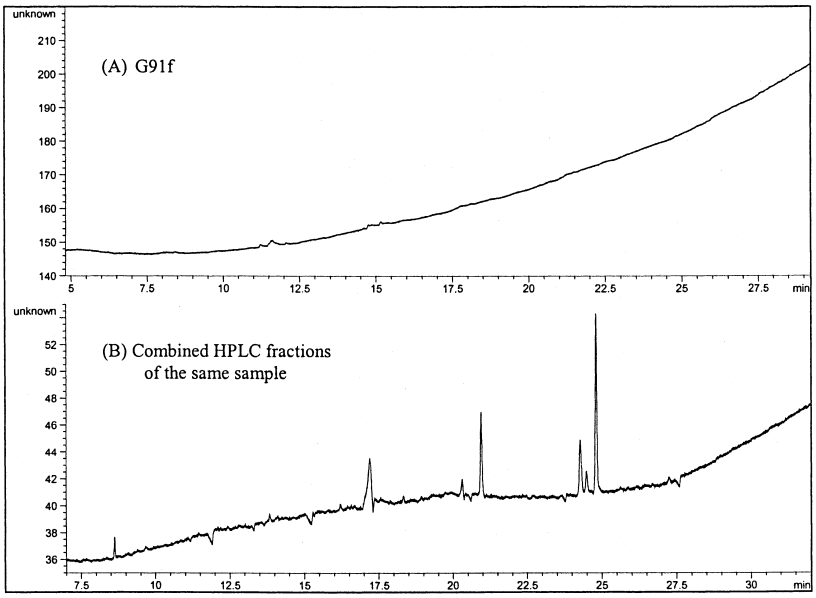

Figure 6. Enrichment effect of HPLC fractionation for a transesterified fillet extract (15 μg Cl/g) prepared from filets of the female fish sampled in the Groundhog River in 1991 (G91f). Column: HP-5.

different. In reversed phase HPLC such as a C-18 column, hydrophobic interaction dominates the elution process, while in GC such as HP-5 (5% phenyl polysilphenylenesiloxane) vapor pressure and polarity are the key parameters for the elution. The matrix compounds co-eluted with chlorinated FAME in HPLC were collected in an HPLC fraction. In the subsequent GC analysis, the chance for a second co-elution is greatly reduced, as the elution mechanism is fundamentally different.

Enrichment via Complexing Mechanisms: A Comparison

Prior to the development of the foregoing HPLC approach, enrichment via complexing mechanisms was tried. The same specimens as used in HPLC fractionation were treated by $AgNO_3$ (silver nitrate) in $EtOH : H_2O$ (1 : 1), $Hg(OAc)_2$ (mercuric acetate), and $(NH_2)_2CO$ (urea). Here is a brief discussion of the results; a full length report was given elsewhere.[8]

Using the silver nitrate method, about 10% of the starting material was removed upon the first treatment and another 10% in the repeated treatment.

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However, little enrichment was achieved for chlorinated FAMEs as evaluated by GC-XSD. This may be due to the fact that this method preferentially removes polyunsaturated FAMEs; but these compounds were not major components of the samples. The mercuric acetate treatment removed about 20% of the material and resulted in measurable enrichment, while it introduced some interference compounds. The urea treatment removed over 90% of the material and achieved very impressive enrichment for certain chlorinated FAMEs. This was expected, because the samples under study contained abundant saturated straight-chain FAMEs, which are preferentially removed by this treatment. However, it was found that not all target chlorinated FAMEs in extracts could be enriched, and the selectivity and extent of enrichment were poorly reproducible. In most cases, large enrichment occurred only of chlorinated FAMEs containing hydrocarbon chains of C_{14} or shorter. Out of several trials using different experimental conditions, there was only one case where dichloro FAMEs of C_{16} and C_{18} were enriched significantly. It was estimated that this treatment resulted in 0 to 30-fold increases in concentrations of chlorinated FAMEs, depending on the matrix composition, structures of individual chlorinated compounds such as chain length and chlorine position, and experimental conditions. In no case, was the concentration of chlorinated FAMEs raised to a level that could be readily detected by FID.

Compared to complexing methods, HPLC enrichment is more effective, selective, reproducible, and predictable, and the specimens enriched by HPLC fractionation contain less interference in subsequent GC analysis.

To see if complexing methods could assist HPLC fractionation, HPLC fractionation of a fish sample (transesterified extract) followed the urea treatment. Though more chlorinated components in HPLC fractions rose to above the detection limit of XSD, not much enrichment was found for target analytes (main EOCl components). This may indicate that the urea treatment did not specifically remove nonchlorinated FAMEs that co-eluted with major chlorinated FAMEs in HPLC elution. Hence, pre-treatment with a complexing method has very limited benefit for HPLC fractionation.

CONCLUSIONS

With the aid of GC-XSD, HPLC fractionation may be the most effective method for removing the bulk of a matrix of fatty acids and enriching target chlorinated compounds for purposes of mass spectrometric identification and confirmation. For the samples under study, the HPLC enrichment enhanced the concentrations of the chlorinated analytes by a factor of 7 to 150, depending on the HPLC elution profile of the analytes against the matrix.

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