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Identification and confirmation of traces of chlorinated fatty acids in fish downstream of bleached kraft pulp mills by gas chromatography with halogen-specific detection

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Abstract

Methyl esters of *threo*-9,10-dichlorooctadecanoic, *threo*-7,8-dichlorohexadecanoic, and *threo*-5,6-dichlorotetradecanoic acids, present in transesterified extracts of filets, gonad, intestinal fat and carcass of white sucker (*Catostomus commersoni*) sampled in receiving waters of bleached kraft pulp mill effluents, were identified by gas chromatography with halogen-specific detection (XSD). Identification was based on (1) a comparison of the retention times of a sample peak with a prospective reference standard on two stationary phases of very different polarities by spiking, and (2) elution behavior of configurational and positional isomers of dichloro fatty acid methyl esters.

Keywords: Fish; Food analysis; Halogen-specific detection; Detection, GC; Kraft pulp; Fatty acids; Organochlorine compounds

1. Introduction

Persistent organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, chlorinated terpenes, chlorinated paraffins, and chlorinated phenolic compounds are well documented environmental contaminants present in fish. However, these compounds represent only a small portion of extractable organochlorine (EOCl) in fish [1]. The identities of major EOCl compounds in fish were unknown until significant work was done by Mu et al. in the past decade [2].

As early as 1972, Lunde studied organobromine and presented findings indicating the presence of acylglycerol-bound, brominated fatty acids in marine fish from the Norwegian coast [3]. In 1977, Stepanichenko et al. reported that 9,10-dichlorooctadecanoic acid was present as a minor lipid in the pathogenic fungus *Verticillium dahliae* [4]. In the same year, White and Hager reported six fatty acid chlorohydrins constituting up to 30% of organic chlorine in extractable lipids from an edible jellyfish [5]. Four of the chlorohydrins were chloro-

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hydroxyoctadecanoic acids and the other two were chlorohydroxyhexadecanoic acids.

Not until 11 years later did environmental scientists speculate that chlorinated fatty acids and lipids could be present in fish exposed to effluent from pulp mills employing bleaching. Hemming and Lehtinen observed that the major components of methylated EOCl of exposed fish have gel permeation chromatographic (GPC) retention times closely matching those of triglycerides and fatty acid methyl esters with the carbon chain containing 18 carbon atoms [6]. Using gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS), Remberger et al. analyzed EOCl from marine sediments contaminated by kraft pulp mill effluents and found that free chlorinated acids consist mainly of chlorinated long-chain alkanoic acids and resin acids [7,8]. Subsequently, Wesén et al. obtained an extract from eel with a high organic chlorine load (1200 ppm) caught at a fiord receiving the effluent from a bleached sulfite pulp mill using chlorine bleaching. By subtracting the amount of EOCl made up of neutral compounds after hydrolysis from the amount of EOCl in neutral compounds after esterification, they concluded that chlorinated fatty acids accounted for 50–85% of EOCl in the eel lipid [9]. Using GC with electrolytic conductivity detection (ELCD) to analyze chlorinated fatty acid methyl esters (chlorinated FAMEs) liberated from lipids by transesterification, it was estimated that these compounds account for 90% of the eel EOCl. By co-injection of a synthetic standard, the largest peak in the ELCD chromatogram was tentatively identified as being methyl 9,10-dichlorooctadecanoate (representing 10% of the EOCl). To confirm the identity by GC–MS, a GC fraction containing the largest ELCD peak was collected by cold trapping [10]. Subsequent MS and GC–MS showed that this methyl dichlorooctadecanoate is present as *threo*- and *erythro*-diastereomers in the eel lipid. Additionally, two diastereomeric forms of methyl tetrachlorooctadecanoate were identified, with the chlorine position remaining undetermined. In order to analyze other less prominent components of the eel EOCl, the concentration of chlorinated FAMEs in the transesterified extract was enriched by consecutive treatments with aqueous silver nitrate and urea, and silica gel thin-layer chromatography [11]. Subsequent study by GC–MS, GC–ELCD and other GC techniques such as linear retention indices, response ratio of ELCD vs. FID, and column difference values indicated the presence of an array of chlorinated FAMEs in the eel lipid sample, including *threo*- and *erythro*-diastereomers of methyl dichlorooctadecanoate, dichlorohexadecanoate and dichlorotetradecanoate [11], isomers of methyl dichlorooctadecenoate, dichlorohexandecenoate and dichlorotetradecenoate, and isomers of methyl tetrachlorooctadecanoate and tetrachlorotetradecanoate [12]. *threo,threo*-Tetrachlorooctadecanoate present in two diastereomeric forms was identified by co-injection of the sample with the synthesized standard and confirmed by high-resolution selected-ion monitoring with ammonia positive chemical ionization [12]. Dichlorotridecanoic acid was later found in eel and herring lipid samples [13].

A comprehensive literature survey of identified naturally-occurring EOCl compounds was presented by Gribble [14], and recently by Dembitsky and Srebnik who focused on halogenated fatty acids [15]. Chlorinated fatty acids have been found not only in marine fish but also in bivalves [16] and lobster [17]. On the other hand, no work on identifying chlorinated fatty acids in freshwater fish has been reported. Also, since the identification work on marine fish was limited to filet extracts, it is not known if the composition of chlorinated fatty acids is tissue specific. Furthermore, although dichlorotetradecanoic acid and dichlorohexadecanoic acid identified in the previous work were claimed to be a 5,6-isomer and a 7,8-isomer, respectively, this has not been proven since the synthesized standards used for identification were all 9,10-isomers and no comparative study on behaviors of positional isomers was conducted. The tentative identifications were largely based on speculation that dichlorotetradecanoic acid in aquatic organisms was derived from β-oxidative metabolism of dichlorohexadecanoic acid, which was in turn produced from β-degradation of dichlorooctadecanoic acid. However, this hypothesis has not been proven by analytical evidence.

These questions called for continuing identification work. To this end, this study focused on identification of chlorinated fatty acids in filets, gonad, intestinal fat and carcass of white sucker sampled in rivers receiving bleached kraft mill effluents. Efforts were made to unambiguously identify exact configurational and positional isomers of

chlorinated fatty acids by synthesizing the exact standards and studying chromatographic behaviors of configurational and positional isomers.

2. Experimental

2.1. Synthesis of (Z)-5-tetradecenoic acid

(*Z*)-5-Tetradecenoic acid, not available directly from chemical suppliers, was synthesized using a procedure based on Jin and Tserng [18]. The synthesis began with a condensation of 5-chloro-1-pentyne (4.08 g) with 1-iodooctane (10.8 g) in the presence of sodium amide (2 g). The resulting 1-chloro-4-tridecyne (8 g, crude) was purified on silica gel. The purified product (1.28 g) was reacted with potassium cyanide (0.41 g) to form 5-tetradecanonitrile (1.3 g, crude), which was hydrolyzed to 5-tetradecynoic acid by a 6-h refluxing in methanol–water (1:1) containing potassium hydroxide. After being purified on silica gel, 5-tetradecynoic acid (224 mg) was reduced to (*Z*)-5-tetradecenoic acid by hydrogen using Lindlar catalyst. The final product was purified on silica gel.

2.2. Nuclear magnetic resonance (NMR) confirmation of (Z)-5-tetradecenoic acid

Both ^{1}H-NMR and total correlation spectroscopy (TOCSY) of the synthesized (*Z*)-5-tetradecenoic acid dissolved in $C^{2}HCl_3$ were recorded at 25 °C on a Varian UNITYplus-500 NMR spectrometer operating at 500 MHz for the proton frequency. Pulse sequence t2pul_longd was used for ^{1}H-NMR, and the relaxation delay was 6 s, pulse angle 63.1°, acquisition time 0.574 s, and spectral width 5296.6 Hz. The data processing involved line broadening 0.1 Hz, and Gauss apodization 0.3 s. Pulse sequence dipsih2o_pfg_wgate_500 was used for TOCSY, and the relaxation delay was 2 s, acquisition time 0.193 s, spectral widths 5296.6 and 5296.6 Hz, and Gauss apodization 0.086 s.

2.3. Chlorination of monounsaturated fatty acids

A series of dichloro fatty acids; *threo*-10,11-dichloroundecanoic, *threo*-5,6-dichlorododecanoic, *threo*-12,13-dichlorotridecanoic, *threo*-5,6-, *threo*-9,10- and *erythro*-9,10-dichlorotetradecanoic, *threo*-10,11-dichloropentadecanoic, *threo*-9,10- and *erythro*-9,10-dichlorohexadecanoic, *threo*-10,11-dichloroheptadecanoic, *threo*-6,7-, *threo*-9,10-, *erythro*-9,10-, *threo*-11,12-, *threo*-13,14- and *threo*-15,16-dichlorooctadecanoic, and *threo*-10,11-dichlorononadecanoic acids, were synthesized from corresponding monounsaturated fatty acids using a method modified from Lyness and Quackenbush [19]. Cl_2 in CCl_4 was added drop by drop into 10 ml $CHCl_3$ solution containing ca. 0.6 g of fatty acid, while being stirred and kept between −10 and −20 °C, until some yellow color persisted. The solvents and excess Cl_2 were removed in a rotary evaporator, and the residual solvents were co-evaporated with hexane. Chlorinated fatty acids were crystallized in 5 ml of hexane in a refrigerator. The recovered product was re-crystallized twice from 3.5 ml hexane. The GC–FID analysis indicated that the purity of the final products was greater than 96%. Chlorohydroxyoctadecanoic, and trichlorohydroxyoctadecanoic and tetrachlorooctadecanoic acids were synthesized previously [20].

2.4. Methylation of chlorinated fatty acids

About 0.6 g of chlorinated fatty acid was methylated in methanol (5 ml) containing 2% (v/v) sulfuric acid at 50 °C for 2 h. The resulting methyl ester was extracted with hexane (3×10 ml) after Nanopure water (10 ml) was added. The combined hexane layers were washed with Nanopure water (10 ml) containing 2% potassium hydrogencarbonate and then with Nanopure water (10 ml), and dried over anhydrous sodium sulfate.

Alternatively, for small-scale preparation, chlorinated fatty acid dissolved in a few drops of methanol was methylated with diazomethane dissolved in ether, prepared from decomposition of 1-methyl-3-nitro-1-nitrosoguanidine [21].

2.5. Sample preparation

Filet samples were taken from white sucker (*Catostomus commersoni*) collected in the fall of 1991 and 1995 from the Mattagami River in northern Ontario downstream from a bleached kraft mill and then frozen [22]. The mill had undergone a process

change from low ClO_2 substitution to 100% ClO_2 (elemental chlorine-free) bleaching in December 1991, added O_2 delignification in 1993 and started biological treatment in 1994. In the present work, the frozen fish filets were ground and homogenized in a blender. After being freeze–dried, the ground filet sample was extracted by pressurized liquid extraction with hexane–acetone (3:1) in an ASE 200 instrument (Dionex) at 55 °C for 10 min and then further at 100 °C for 5 min [23]. Extracts in the organic solvent were washed in a separatory funnel with Nanopure water (acidified to pH 3.0 by H_2SO_4) and dried over anhydrous Na_2SO_4. The low-molecular-mass portion of the extracts, presumably consisting mainly of chlorinated pesticides and PCBs, was removed by GPC using a pre-flushed 60×2.5 cm glass column packed with Bio-Beads SX3 (cyclohexane as an eluent) [24]. An aliquot (~0.6 g) of the high-molecular-mass fraction (a cutoff of $M_r \approx 230$, estimated from the elution profile of a mixture of triolein and biphenyl) was dissolved in 1–2 ml of toluene and transesterified in 5 ml of methanol containing 2% (v/v) of H_2SO_4 at 50 °C overnight [25]. The subsequent workup followed the same procedure as in Section 2.4. The resultant transesterified extracts were labelled M91F, M95F and M95M with the first letter representing the Mattagami River; two-digit number, the sampling year; and the ending letter, the fish gender (no male fish sample available for the 1991 sampling).

The GPC high-molecular-mass fractions of extracts of gonad, intestinal fat and carcass from fish of the same species were donated by Sonnenberg. These fish were sampled in 1993 in the St. Maurice River downstream from a bleached kraft mill in southern Québec, where 50–70% ClO_2 substitution was employed for bleaching, and the effluent was not biologically treated. The extracts were obtained from these samples by Polytron extraction in cyclohexane–isopropanol (1:1) [24]. The subsequent treatment leading to transesterified products was the same as for the filet extracts of the Mattagami River fish.

Reference samples were prepared from filets taken from fish sampled in reference rivers close to the sampling site in the Mattagami River and various tissues from fish sampled in the St. Maurice River upstream from the mill, using the same procedures as for their corresponding exposed fish.

2.6. GC–halogen-specific detection (XSD) analysis

GC chromatograms were recorded with a HP 6890 GC instrument equipped with an OI Analytical 5360 halogen-specific detector. Standards and samples were dissolved in isooctane and the injection volume was 1 μl. The injector was operated at 270 °C in the splitless mode. The column was either a HP-5MS or a DB-WAX column (30 m×0.25 mm, 0.25 μm) and 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 detector was operated at 1100 °C with an air flow of 40 ml/min.

Retention times in chromatograms obtained in separate sequences run on different days might be shifted. When these chromatograms were presented together in a figure, they were aligned according to linear relationships of retention time between sequences established with a standard solution containing a mixture of chlorinated FAMEs run in each sequence [26].

3. Results and discussion

3.1. Verification of (Z)-5-tetradecenoic acid by NMR

The structure of the synthesized (*Z*)-5-tetradecenoic acid was verified by NMR. Fig. 1 is an H^1-NMR spectrum of this purified product. The assignment of the peaks in the spectrum was verified by TOCSY (not shown) and indicated in the molecular formula shown in Fig. 1. The inset in Fig. 1 shows a split pattern characteristic of the *cis* configuration of alkene: J_{ab}=~10 Hz, and J_{ae} and J_{bd}=~7 Hz. In comparison, the *trans* configuration would result in J_{ab}=17 Hz [27]. The integral of each assigned proton is given in Table 1. The number of protons bound to each carbon atom shown in the last column of Table 1 is in agreement with the assignment indicated in the molecular formula illustrated in Fig. 1. Since the number of H_f is 2 and that of H_g is 12, it follows that m=1 and n=6. This confirms that the double bond of the synthesized monoenoic acid is at C_5.

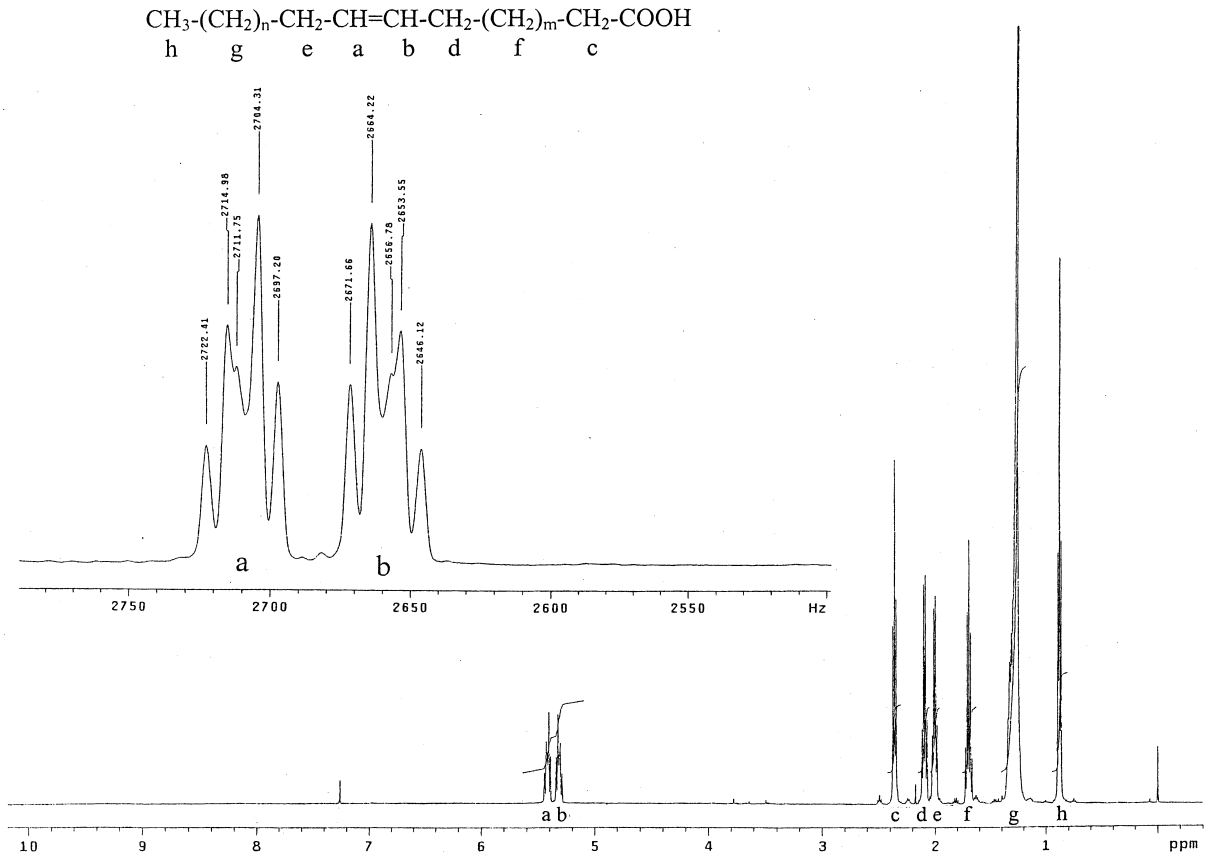

Fig. 1. ^{1}H-NMR spectrum of final product in synthesis of (*Z*)-5-tetradecenoic acid (500 MHz, $C^{2}HCl_3$).

3.2. Chromatographic properties of chlorinated fatty acid methyl esters

Synthesized dichloro FAMEs were well separated from each other according to the length of the carbon chain. As is seen in Fig. 2, elution time increases as the carbon chain length of dichloro FAMEs increases. Both nonpolar (HP-5) and polar (DB-WAX) GC columns give a good linear correlation between the retention time and the number of carbon atoms in

Table 1
Analysis of ^{1}H-NMR signals (Fig. 1)

Peak assignment[a]	Splitting	Integral region	Integral	No. protons
a	Doublet of triplet	5.64–5.09	2.23	1
b	Doublet of triplet			1
c	Triplet	2.43–2.30	2.09	2
d	Doublet of triplet	2.16–2.05	2.01	2
e	Doublet of triplet	2.05–1.96	2.00	2
f	Quintet	1.76–1.63	2.03	2
g	Multiplet	1.41–1.18	12.56	12
h	Triplet	0.95–0.81	3.07	3

[a] See the molecular formula given in Fig. 1.

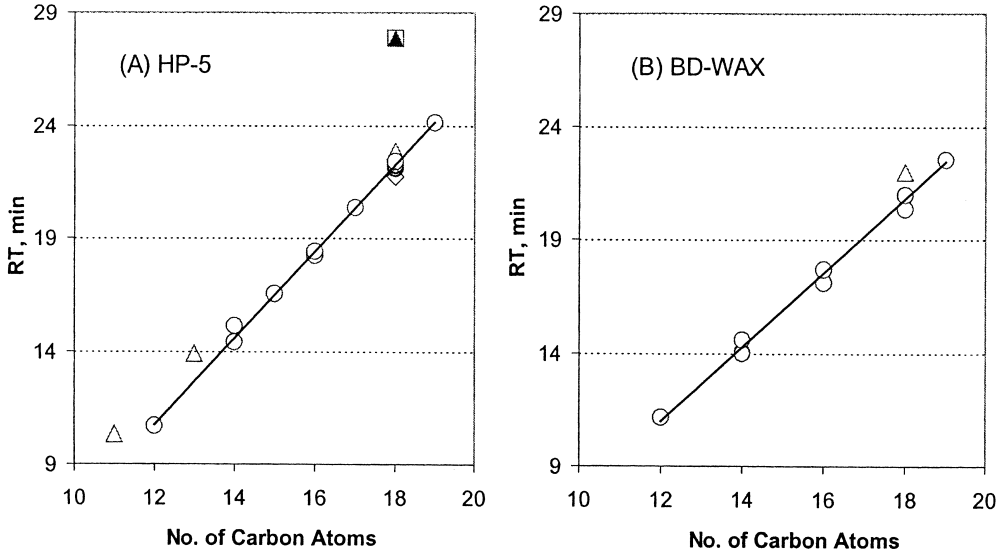

Fig. 2. Linear relationship between the number of carbon atoms of dichloro FAMEs and GC retention time. ○ Dichloro FAMEs with vicinal chlorine atoms located around the middle of the straight carbon chain: *threo*-5,6-dichlorododecanoate, *threo*-5,6-, *erythro*-9,10 and *threo*-9,10-dichlorotetradecanoates, *threo*-10,11-dichloropentadecanoate, *erythro*- and *threo*-9,10-dichlorohexadecanoate, *threo*-10,11-dichloroheptadecanoate, *erythro*-9,10-, *threo*-9,10-, *threo*-11,12-, *threo*-12,13- and *threo*-13,14-dichlorooctadecanoates, and *threo*-10,11-dichlorononadecanoate; △ Dichloro FAMEs with vicinal chlorine atoms located at or close to the end of the straight carbon chain: 10,11-dichloroundecanoate, 12,13-dichlorotridecanoate, and *threo*-15,16-dichlorooctadecanoate; ◇ methyl chlorohydroxyoctadecanoate, □ methyl trichlorohydroxyoctadecanoate; ◆ methyl tetrachlorooctadecanoate.

dichloro FAMEs having vicinal chlorine atoms located around the middle of the chain. Incorporation of additional chlorine atoms, as in the case of tetrachloro FAMEs, results in a much longer retention time. A hydroxyl group seems to have the same effect on the elution time as does chlorine since both tetrachloro and trichlorohydroxy FAMEs of C_{18} are eluted at about the same time (Fig. 2A).

threo- and *erythro*-Isomers can be separated from each other by GC (Fig. 3), which agrees with earlier results reported by Mu et al. [12]. The chromatograms show that DB-WAX has a better resolution than HP-5. On both columns, the *erythro* isomers (compounds b, d and g) are eluted before their corresponding *threo* isomers (compounds c, e and h). Indeed, when these two diastereomeric forms are separated as two groups, a very high degree of linear relationship is established between the number of carbon atoms in dichloro FAMEs and the retention time (Fig. 4).

Some positional isomers can be separated by GC as well. On both columns, dichloro FAMEs with vicinal chlorine atoms located closer to the carboxylic group are eluted earlier. The separation of peaks a and c in the chromatograms shown in Fig. 3 indicates that it is likely possible to separate *threo*-5,6-, 7,8- and 9,10-isomers of dichlorotetradecanoic acid methyl esters since there is some space between peaks a and c for insertion of the 7,8-isomer (if the *erythro* isomer is not considered in the HP-5 chromatogram). But this would not be the case for positional isomers of dichlorooctadecanoic acid methyl ester as indicated by clustering of the 6,7-, 9,10- and 13,14-isomers (peaks f, h and i). Thus, the increase in the length of the carbon chain tends to diminish chromatographic differences of diastereomers and, to a greater extent, of positional isomers. Clustering of peaks f, h and i indicates that it is more difficult to separate those positional isomers whose vicinal chlorine atoms are located around the middle of the hydrocarbon chain. Dichloro FAMEs which have vicinal chlorine atoms residing closer to the chain terminus (e.g., peak j) are easier to separate from other positional isomers.

Different chromatographic properties between a nonpolar and a polar column in eluting chlorinated

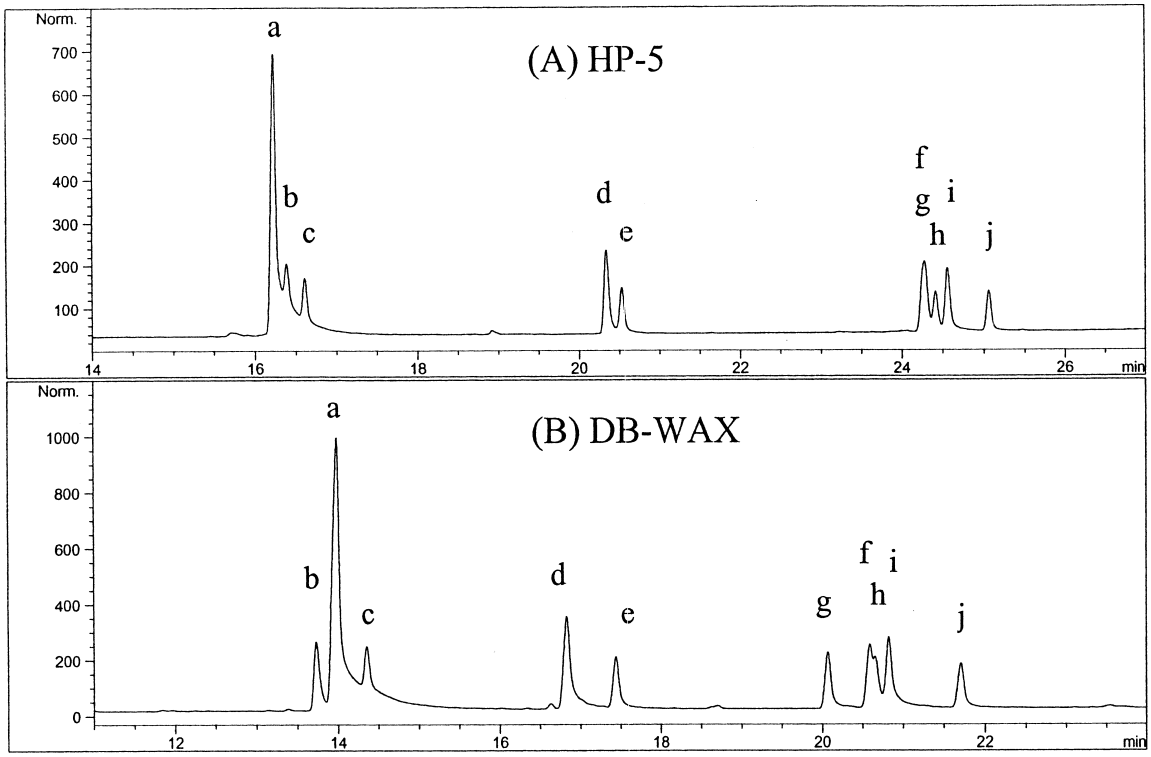

Fig. 3. Difference in GC retention times between *erythro*- and *threo*-isomers and positional isomers of synthesized dichloro FAMEs, and effects of polarities of the GC stationary phase on relative retention times of dichloro FAMEs. Peaks: a, b, c=*threo*-5,6-, *erythro*-9,10- and *threo*-9,10-dichlorotetradecanoate; d, e=*erythro*-9,10- and *threo*-9,10-dichlorohexadecanoate; f, g, h, i, j=*threo*-6,7-, *erythro*-9,10-, *threo*-9,10-, *threo*-13,14- and *threo*-15,16-dichlorooctadecanoate. Note that peaks f and g overlapped with each other in the chromatogram from the HP-5 column.

FAMEs are demonstrated in Fig. 3. The relationship between retention time and location of vicinal chlorine atoms and changes in this relationship resulting from using a different type of column are illustrated in Fig. 5. In addition to spacing changes between peaks, the elution order of some dichloro FAMEs (e.g., peaks a and b, and f and g) may be altered when a GC column of very different polarity is employed. This property was used in this work for confirming identified chloro FAMEs.

3.3. Identification of chlorinated fatty acids in transesterified extracts by GC–XSD

Since XSD is highly specific for organohalogen including chlorinated fatty acids [28,29], the advantage of using XSD as a GC detection method is that chlorinated fatty acids present at trace levels in a sample dominated by nonchlorinated fatty acids can be detected without resorting to pre-enrichment of the analytes. The GC retention times of a series of synthesized dichloro fatty acids were compared with those of chlorinated components of transesterified fish extracts in GC–XSD analysis. It appears that major peaks from the transesterified extracts from fish sampled downstream from bleached kraft pulp mills are attributable to methyl dichlorotetradecanoate, dichlorohexadecanoate and dichlorooctadecanoate. However, because EOCl compounds were present at trace levels in transesterified fish extracts, the elution process of chlorinated species might be disturbed by co-eluted or closely eluted nonchlorinated matrix species that were present in high concentrations. A simple comparison of retention time between standards and sample analytes in chromatograms from separated runs would thus

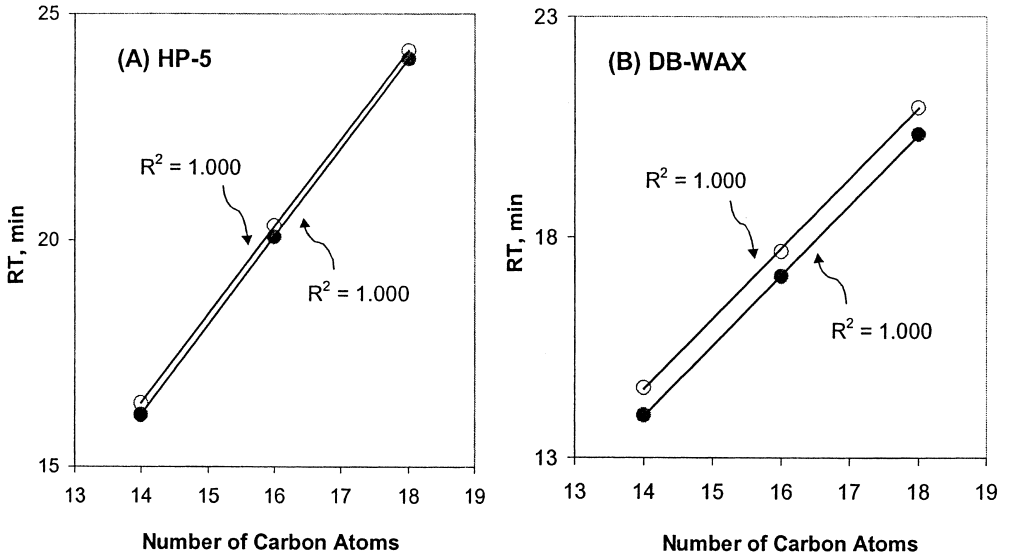

Fig. 4. Linear relationship between the number of carbon atoms of dichloro FAMEs and GC retention time in two homologous series. ○ *threo*-9,10-Dichloro FAMEs; ● *erythro*-9,10-dichloro FAMEs.

not be rigorous. To have standards and sample analytes eluted through a GC column exactly in the same elution environment, samples were spiked with standards to test if their peaks are superposed. Fig. 6 is a comparison of the GC–XSD chromatograms, before and after spiking, of the transesterified extracts from female fish sampled in Mattagami River downstream from a bleached kraft mill in 1991 (M91F) and in 1995 (M95F), and from male fish sampled downstream in 1995 (M95M). Similar

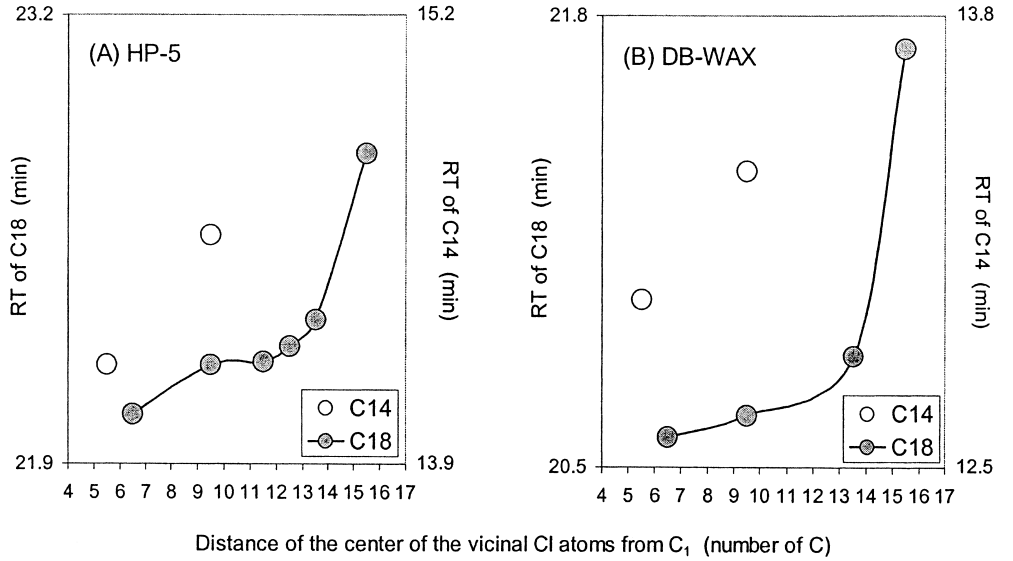

Fig. 5. GC retention time of two series of dichloro FAMEs, *threo*-dichlorotetradecanoate (C_{14}) and *threo*-dichlorooctadecanoate (C_{18}), as a function of distance of the center of the vicinal Cl atoms from the carboxylic group.

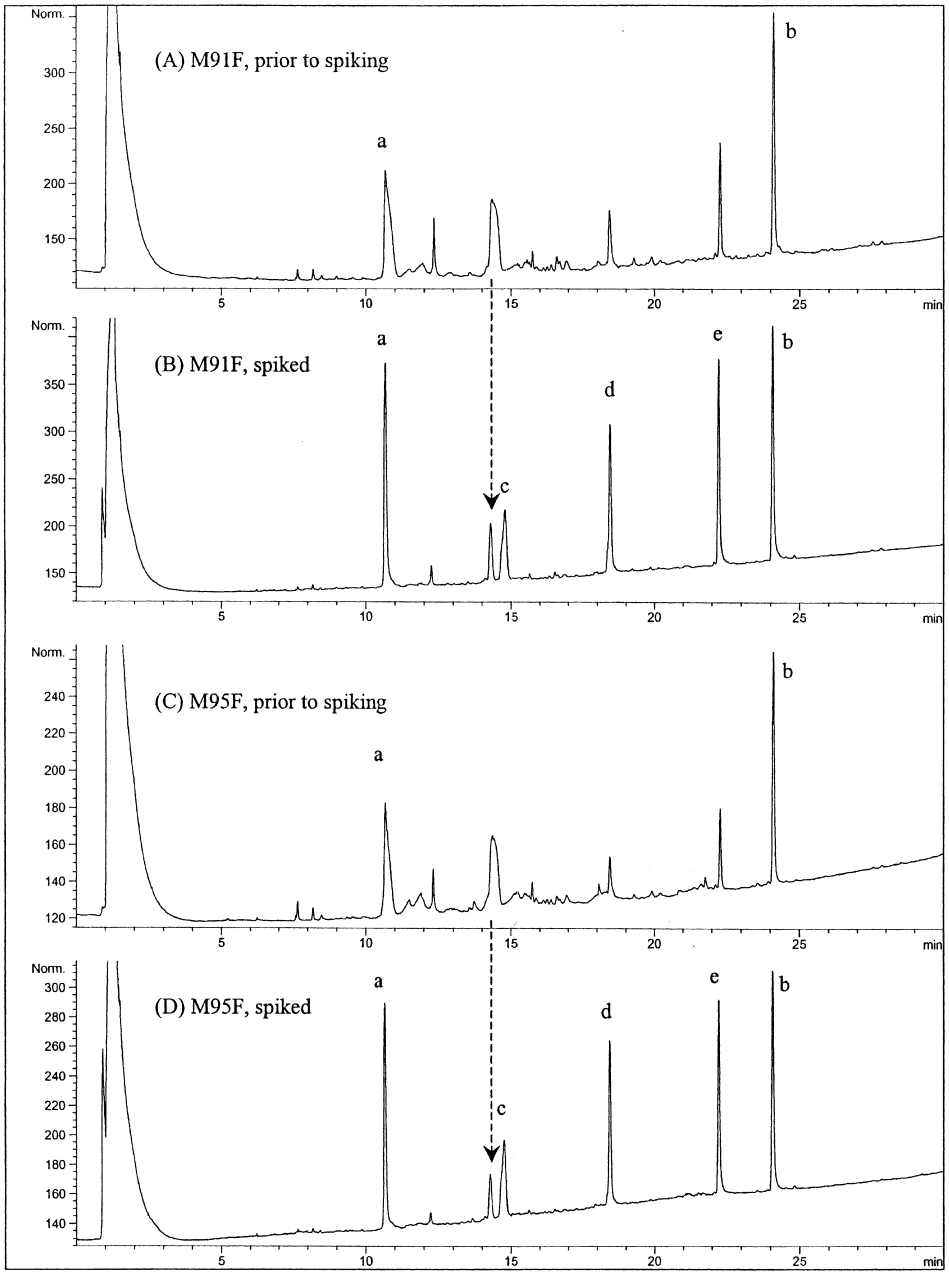

Fig. 6. GC–XSD chromatograms of transesterified extracts of filets from fish sampled in the bleached kraft mill effluent recipient water (column: HP-5). Internal standards: methyl esters of (a) *threo*-5,6-dichlorododecanoic acid and (b) *threo*-10,11-dichlorononadecanoic acid. Standards for spiking: methyl esters of (c) *threo*-9,10-dichlorotetradecanoic acid, (d) *threo*-9,10-dichlorohexadecanoic acid, and (e) *threo*-9,10-dichlorooctadecanoic acid. M91: Sampled in Mattagami River downstream from the bleached kraft mill in 1991; M95: sampled at the same location in 1995. F: female fish; M: male fish. Note: Broadening and distorting of two prominent peaks in the chromatograms prior to spiking were due to interference of overloaded co-eluted nonchlorinated matrix components [29]. Thanks to dilution of matrix components by the spiking solution, the peak abnormality of these two chlorinated compounds was eliminated in the chromatograms following spiking. However, peak c was broad, probably because one or more matrix compounds co-eluted with this spiked peak were still overloaded.

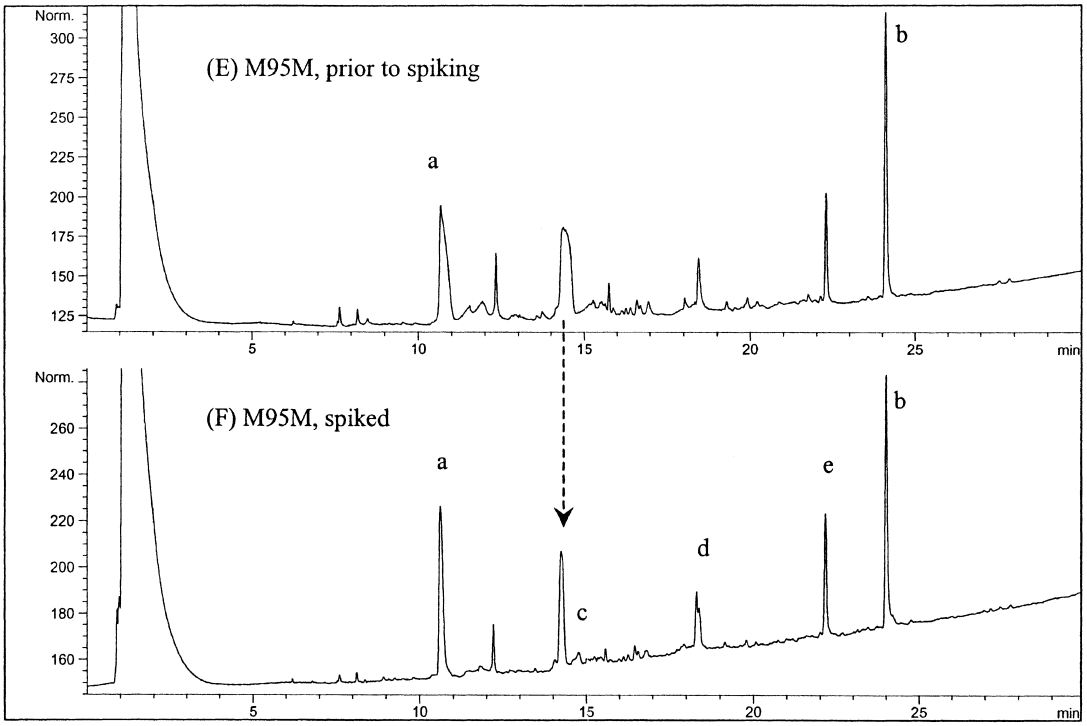

Fig. 6. (*continued*)

chromatograms were obtained for the spiked transesterified extracts of gonads, intestinal fat and carcass from the white sucker fish sampled in St. Maurice River downstream from a bleached kraft pulp mill using 50% ClO_2 substitution (Fig. 7). In these chromatograms there are two internal standard peaks (a and b) corresponding to methyl *threo*-5,6-dichlorododecanoate and *threo*-10,11-dichlorononadecanoate. Preliminary chromatographic runs of any transesterified fish extracts under study had shown that there were no appreciable XSD responses in the region of retention time where these internal standards would be present. The sample solutions were spiked with a solution consisting of five standards in approximately equal molarity: *threo*-9,10-dichloro FAMEs of C_{14}, C_{16} and C_{18}, and the internal standards. As we see, the synthesized methyl dichlorooctadecanoate (e) coincides in retention time with a major sample component, but the synthesized methyl *threo*-9,10-dichlorotetradecanoate (c) apparently does not. The synthesized methyl *threo*-9,10-dichlorohexadecanoate (d) seemingly co-elutes perfectly with a major sample component, a close look reveals that it does not (Fig. 8). It is discernable in Fig. 8 that the methyl *threo*-9,10-dichlorohexadecanoate (d) spiked is consistently slightly behind sample peak d′, whereas the methyl 9,10-dichlorooctadecanoate (e) spiked is perfectly superimposed on sample peak e′ (Fig. 8C and E).

According to a literature survey conducted by McKague et al. [30], 9,10-dichlorooctadecanoic acid was the only reported chlorinated fatty acid that was found in the chlorine bleaching effluent. It is known that the major monoenoic fatty acid in the wood is *cis*-9-octadecenoic acid [31], so it is reasonable that *threo*-9,10-dichlorooctadecanoic acid would be produced in the chlorine bleaching process. Due to its hydrophobicity and structural similarity to biogenic fatty acids, it could be accumulated in biota when it is discharged into the receiving water. Therefore, sample peak e′ is identified as being *threo*-9,10-dichlorooctadecanoate.

Dichlorotetradecanoic and dichlorohexadecanoic acids have not been reported in bleaching effluents.

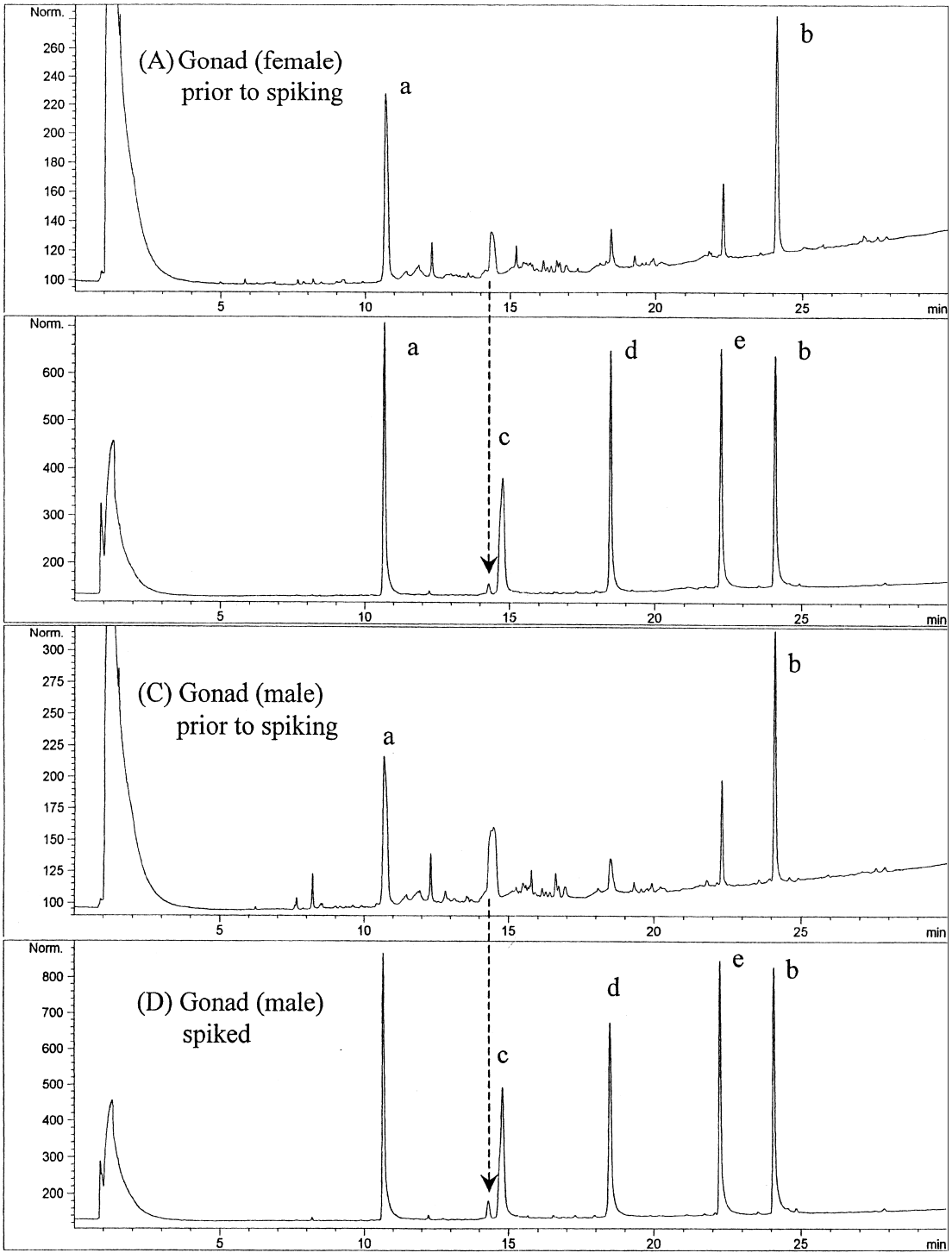

Fig. 7. GC–XSD chromatograms of transesterified extracts of different tissues from fish sampled in May 1993 in the St. Maurice River downstream from a bleached kraft mill using 50% ClO_2 substitution (column: HP-5). The internal standards (a and b) and the standards for spiking are as in Fig. 6. Also see the note in Fig. 6.

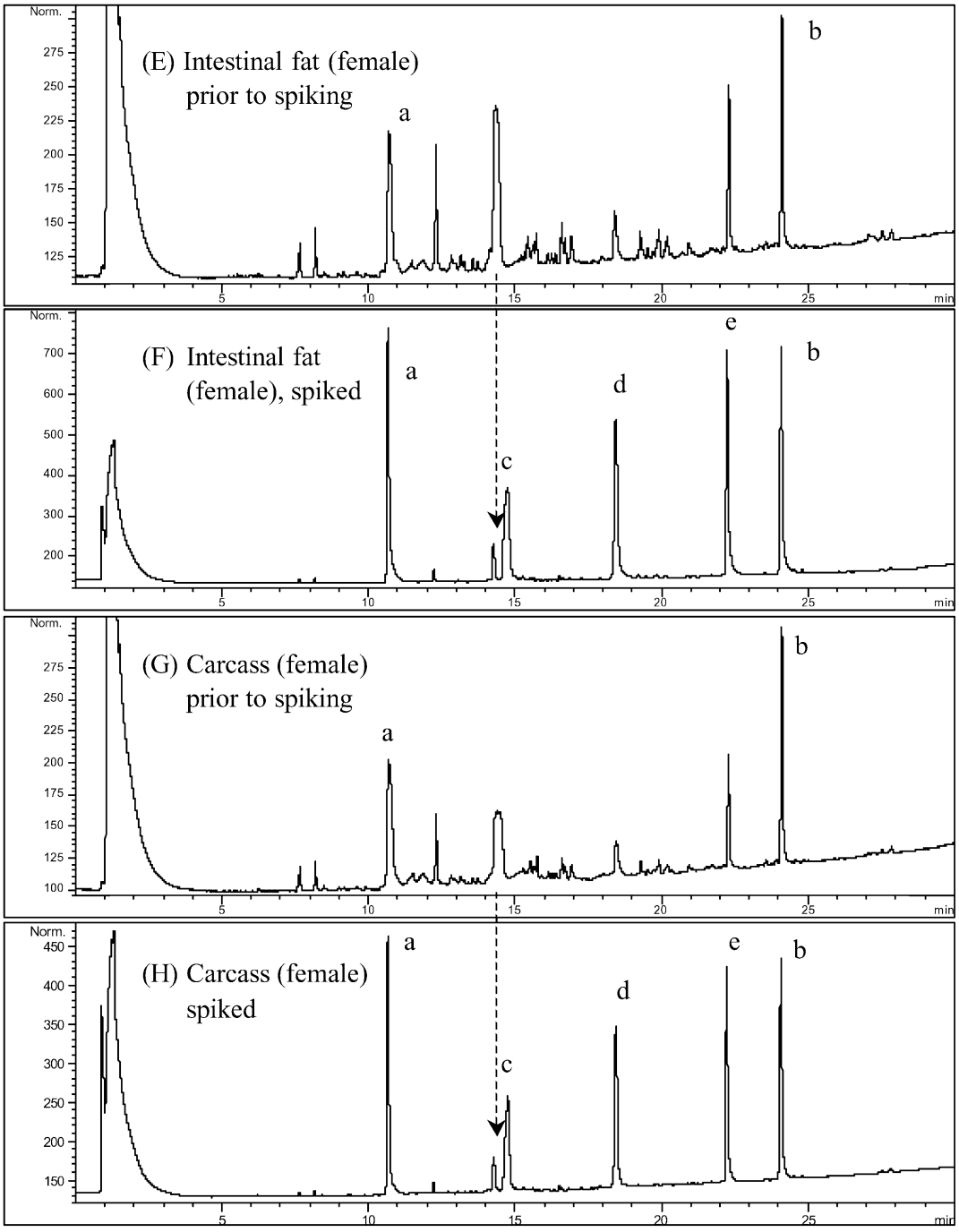

Fig. 7. (*continued*)

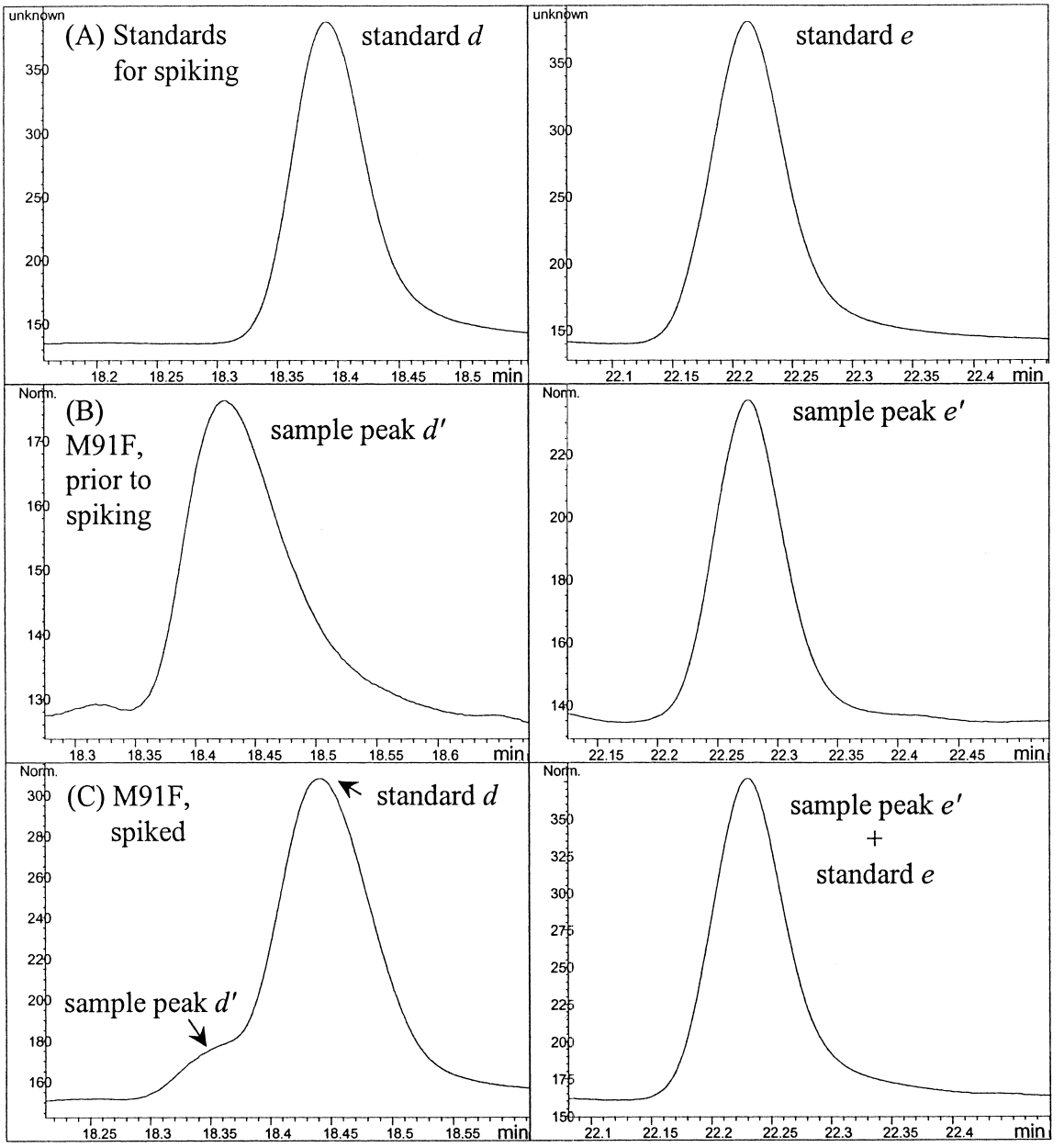

Fig. 8. Enlarged portions of GC–XSD chromatograms, showing a mismatch between sample peak d′ and standard d (methyl *threo*-9,10-dichlorohexadecanoate) and a perfect match between sample peak e′ and standard e (methyl *threo*-9,10-dichlorooctadecanoate). (B–E) Portions of the chromatograms shown in Fig. 6A, B, E and F, respectively.

Though this can not be taken as evidence for their absence in bleaching effluents, their concentration in the effluents is therefore expected to be low if there is any. On the other hand, a study of the metabolism of halogenated fatty acids in rats has concluded that 9,10-dichlorooctadecanoic acid can be degraded via β-oxidation to 7,8-dichlorohexadecanoic acid, which is in turn further degraded into 5,6-dichlorotet-

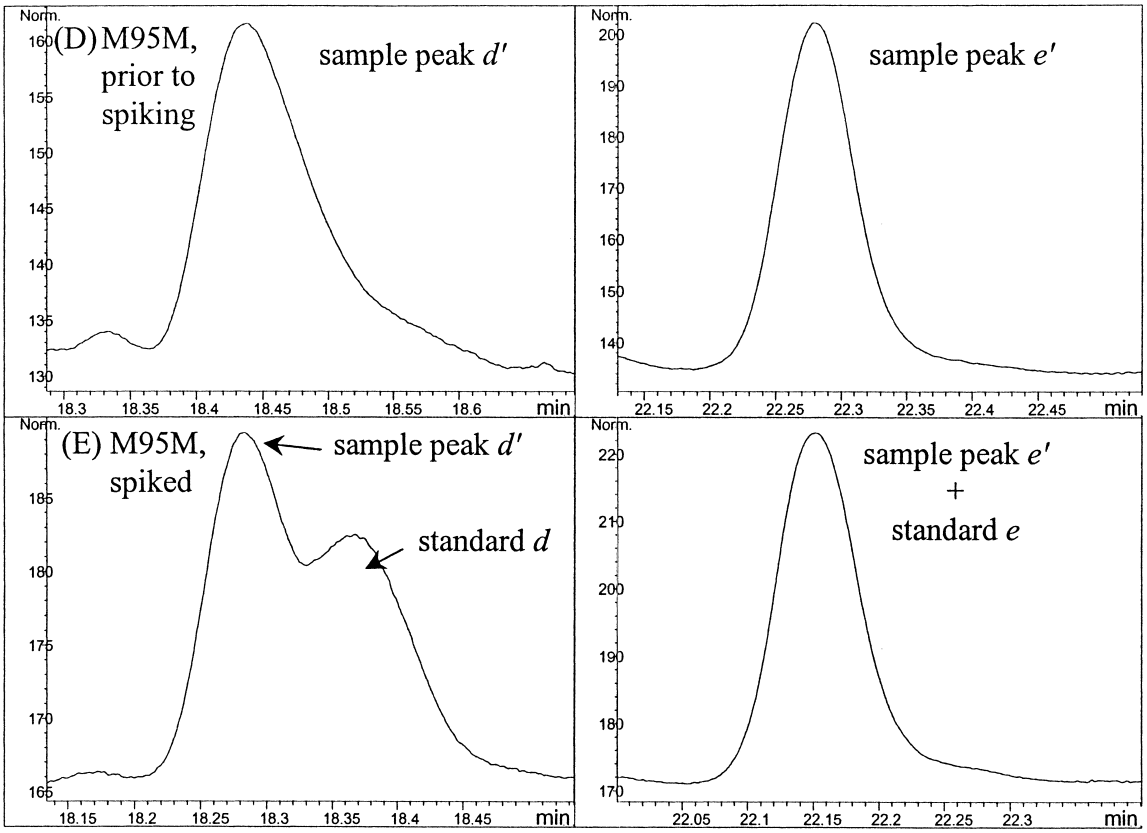

Fig. 8. (*continued*)

radecanoate [32]. The β-oxidation seems to occur also in cultured human cells [33]. It is thus possible that the same metabolism mechanism may also be operative in fish for degrading chlorinated fatty acids. Therefore, a working hypothesis is that two other major chlorinated fatty acid methyl esters in the transesterified fish extracts are methyl *threo*-5,6-dichlorotetradecanoate and 7,8-dichlorohexadecanoate. The chlorinated compound eluted just before standard c (methyl *threo*-9,10-dichlorotetradecanoate) in Figs. 6 and 7 is thus likely to be methyl *threo*-5,6-dichlorotetradecanoate. The other major chlorinated component whose difference in retention time from standard d (methyl *threo*-9,10-dichlorohexadecanoate) can be discerned only in magnified chromatograms (Fig. 8) could then be the *threo*-7,8-isomer of dichlorohexadecanoate.

Hence, synthesized standards of these positional isomers are valuable for identification and confirmation. (Z)-5-tetradecenoic acid, which can be easily chlorinated to form *threo*-5,6-dichlorotetradecanoic acid, was made from 5-chloro-1-pentyne and 1-iodooctane and confirmed by NMR (Section 3.1). Fig. 9 shows the GC–XSD chromatograms of the transesterified fish extracts before and after spiking with the synthesized methyl *threo*-5,6-dichlorotetradecanoate. The amount of the synthesized standard added to the samples was approximately the same as the amount of the target compound present in the samples. As we see in Fig. 9, the standard was exactly superimposed on the most abundant chlorinated component in the transesterified fish extracts, leading to a doubling of the peak intensity. A close look at these chromatograms does not reveal any change in characteristics of peak before and after spiking (Fig. 10). The peaks in Fig. 10C and D look broadened and the peak tops flattened. But this abnormality occurred both before and after spiking

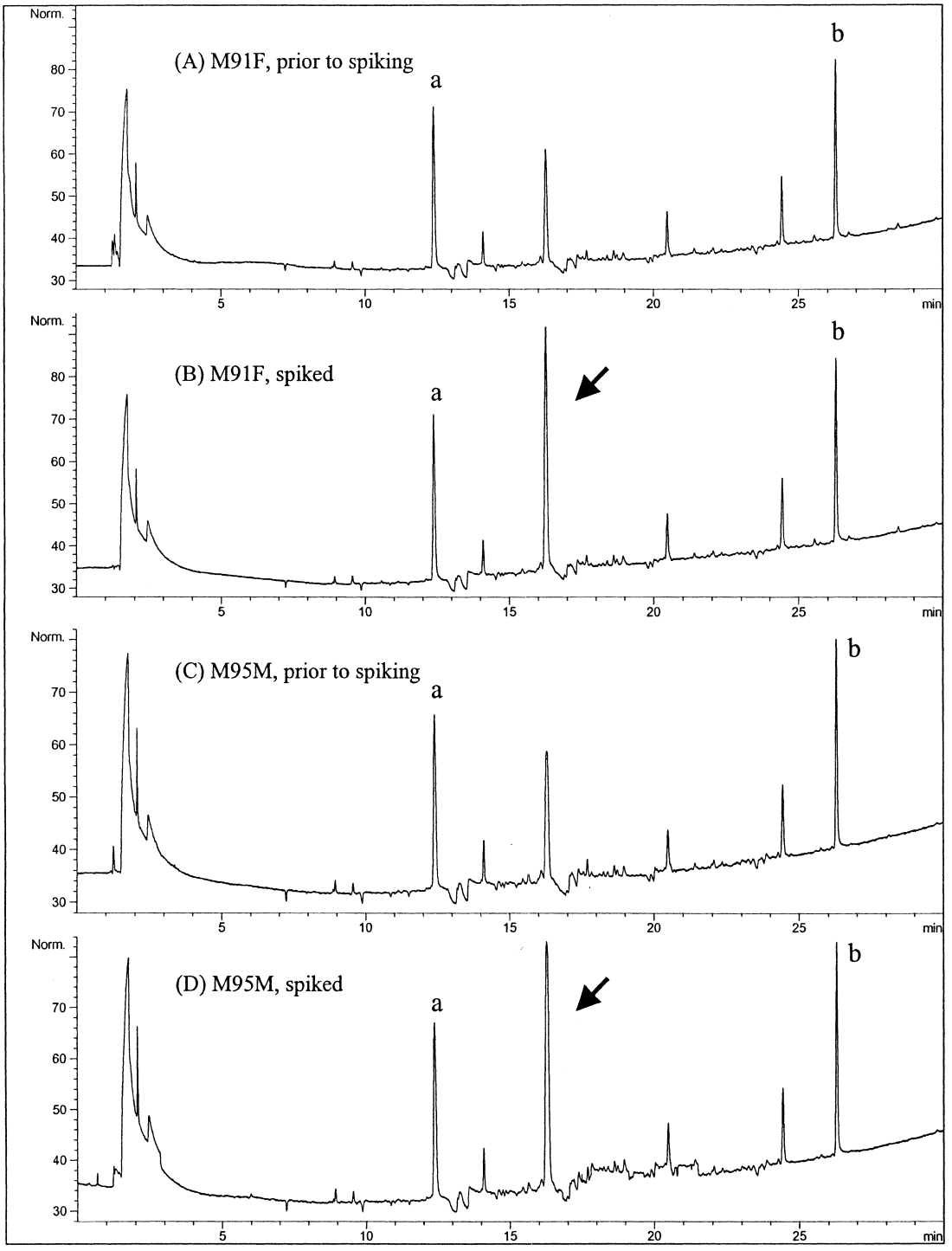

Fig. 9. Identification of methyl *threo*-5,6-dichlorotetradecanoate in transesterified filet extracts by GC–XSD with the authentic standard (column: HP-5). The standard for spiking: methyl *threo*-5,6-dichlorotetradecanoate. Internal standards (a and b) and sample labeling as in Fig. 6.

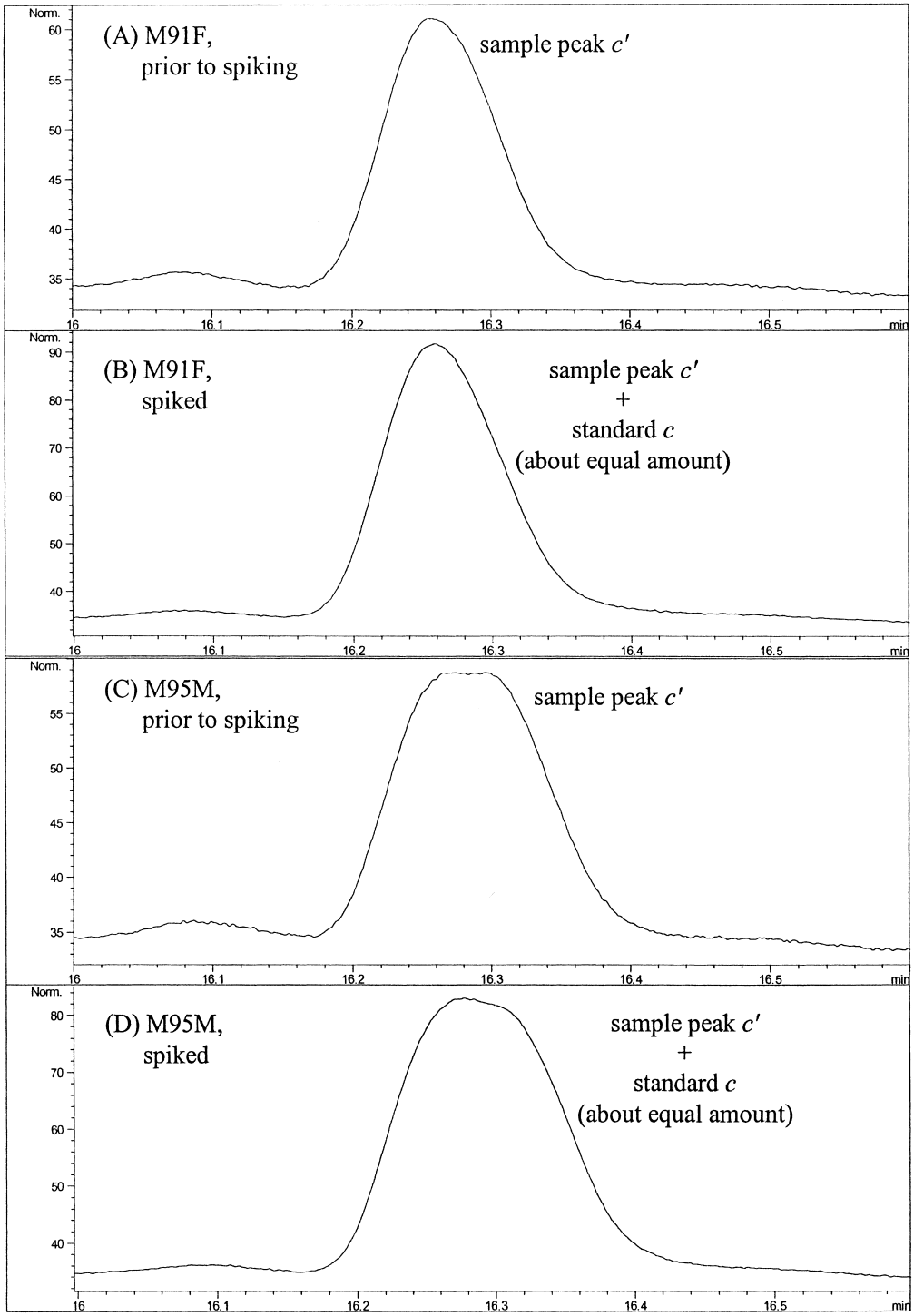

Fig. 10. Enlarged portions of GC–XSD chromatograms from Fig. 9, showing a perfect match between sample peak c′ and standard c (methyl *threo*-5,6-dichlorotetradecanoate).

and thus was likely caused by the interference of the matrix in this particular sample. Therefore, sample peak c′ is identified as being *threo*-5,6-dichlorotetradecanoate.

No synthesis of *threo*-7,8-dichlorohexadecanoic acid was made. Nevertheless, sample peak d′ can be relatively convincingly assigned to be this compound. First of all, the identification of *threo*-5,6-dichlorotetradecanoic acid in the fish extracts supports the β-oxidative metabolism, therefore the identification of *threo*-7,8-dichlorohexadecanoic acid can be rationalized since it is the parent compound of *threo*-5,6-dichlorotetradecanoic acid in the β-oxidative pathway. Second, the analyte peak barely preceding methyl *threo*-9,10-dichlorohexadecanoate in the chromatograms is in agreement with chromatographic behaviors expected for the 7,8- and 9,10-isomers. Based on the study of positional isomers on nonpolar GC columns (Section 3.2), it is expected that a dichloro FAME would elute ahead of those positional isomers that have vicinal chlorine atoms positioned farther away from the carboxylic group, and that positional isomers of a long chain dichloro FAME having their vicinal chlorine atoms located around the middle of the carbon chain would be hardly separated.

Figs. 6 and 7 reveal that chromatograms of transesterified fish extracts under study are very similar regardless of the tissue and have the same characteristic peaks.

In general, there were no appreciable XSD responses in the GC chromatograms of reference samples (not shown). In particular, no peaks were observed at the retention times where three identified dichloro FAMEs would appear.

3.4. Confirmation by GC–XSD

A simple approach of confirming the identified dichloro FAMEs is to run the samples on a different GC column whose polarity is very different from that of the GC column used in prior identification (HP-5). The stationary phase of HP-5 (5% phenyl–95% methyl polysiloxane) is basically nonpolar. Thus, DB-WAX (polyethylene glycol), a typically polar GC column, was selected for this purpose. The GC–XSD chromatograms (DB-WAX) of the transesterified fish extracts before and after spiking with the synthesized standards are illustrated in Fig. 11. As we see in Fig. 11, two major components from the transesterified fish extracts were co-eluted with standards c and e, i.e., methyl *threo*-5,6-dichlorotetradecanoate and 9,10-dichlorooctadecanoate, and the spiking increased the intensity of their peaks accordingly. Since the co-elution of the analytes and their respective standards occurred in both columns that have very different polarities, their identification was thus confirmed. A sample peak was seemingly co-eluted with standard d (methyl *threo*-9,10-dichlorohexadecanoate), but it is noted that the spiking raised its intensity very little—far less than it would expected were they the same compound. Though the mismatch of 9,10-dichlorohexadecanoate spiked with the target sample peak is less obvious in the DB-WAX chromatogram than in the HP-5 chromatogram, it is discernable in the magnified chromatograms by examining changes in the peak shape that occurred as the molar ratio of the standard spiked vs. the sample component was varied. An example is illustrated in Fig. 12. The sample peak is ahead of the standard peak in retention time, as expected from the chromatographic behaviors of positional isomers illustrated in Section 3.2. This experiment thus provides an additional hint that the analyte eluted closely with the synthesized methyl *threo*-9,10-dichlorohexadecanoate is a 7,8-isomer.

4. Conclusions

threo/*erythro* Isomers of dichloro FAMEs can be separated by GC. Separation of neighboring positional isomers can be readily achieved by GC for those having dichloro groups present closer to either of both ends of the carbon chain or for those having shorter carbon chains such as dichlorotetradecanoate. Chromatographic behaviors of these isomers are predictable. The GC–XSD chromatograms of transesterified extracts from different tissues of the exposed fish are remarkably similar. *threo*-5,6-Dichlorotetradecanoic and 9,10-dichlorooctadecanoic acids were identified and confirmed by GC–XSD with two columns of very different polarities and using synthesized authentic standards as spikes. This identification provides evidence for the β-oxidative metabolism of dichlorooctadecanoic acid in fish. The

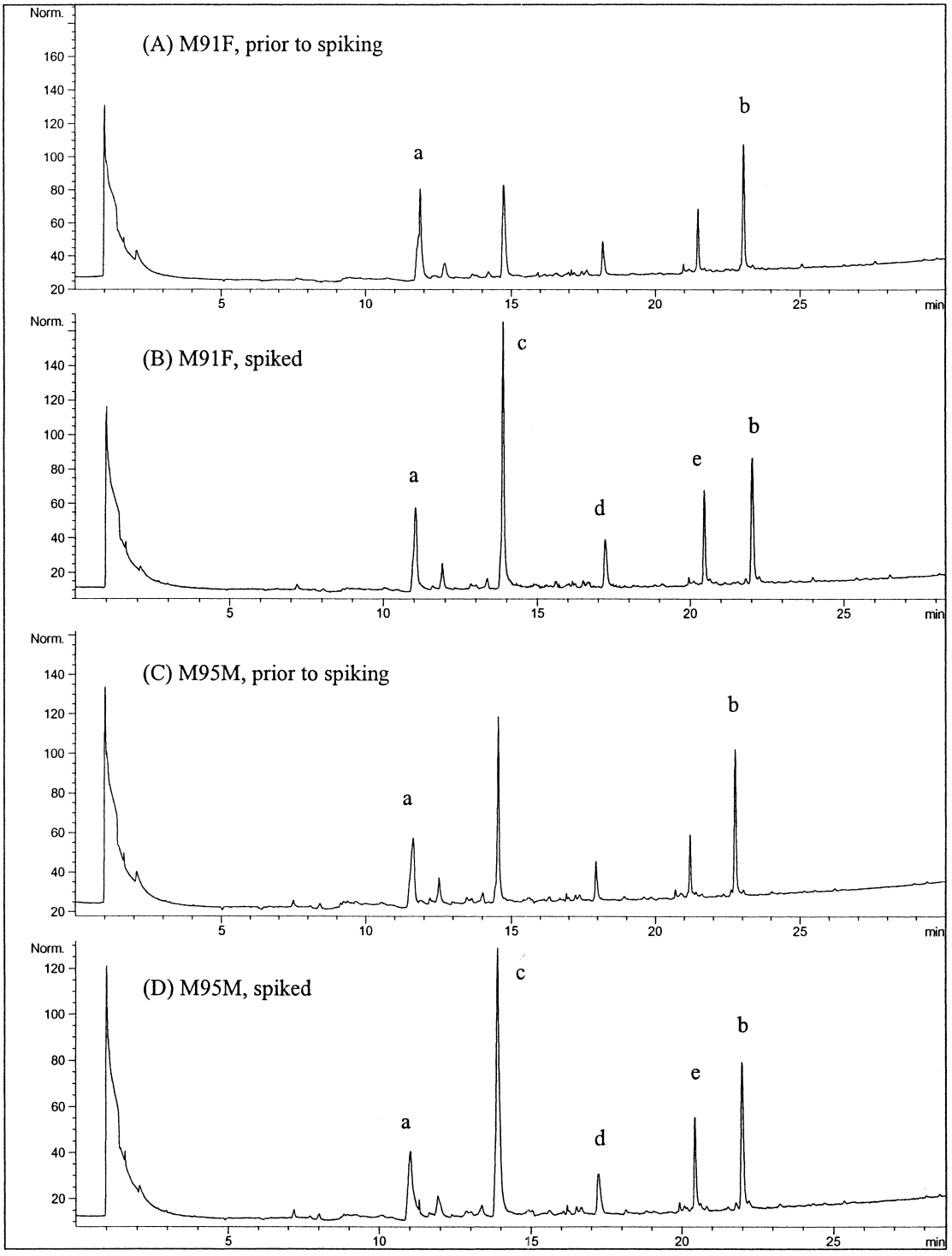

Fig. 11. Confirmation of identified dichloro FAMEs using a different column (DB-WAX). Standards for spiking: methyl esters of (c) *threo*-5,6-dichlorotetradecanoic acid, (d) *threo*-9,10-dichlorohexadecanoic acid and (e) *threo*-9,10-dichlorooctadecanoic acid. Internal standards and sample labeling as in Fig. 6.

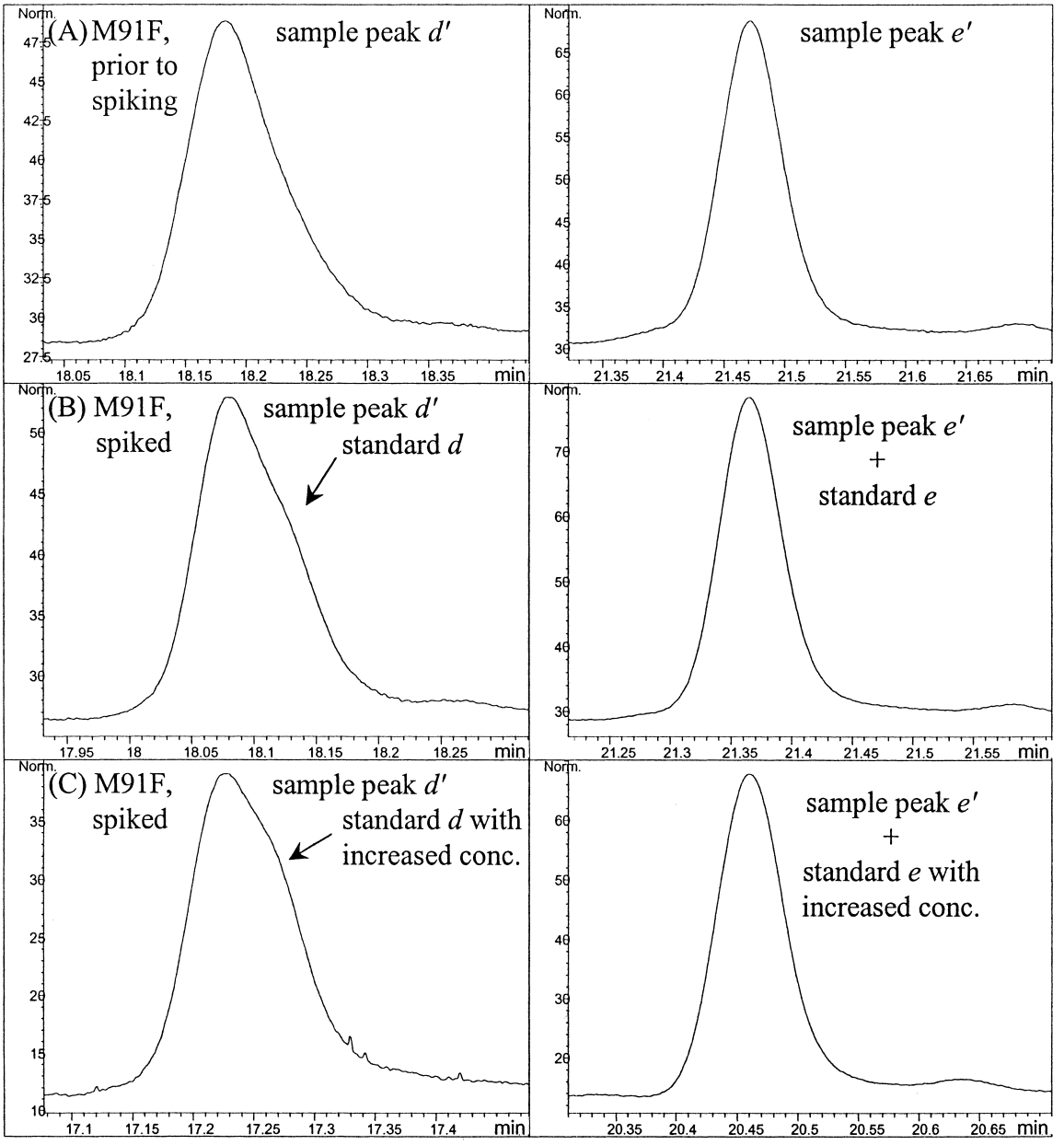

Fig. 12. Enlarged portions of GC–XSD chromatograms showing a mismatch between sample peak d′ and standard d (methyl *threo*-9,10-dichlorohexadecanoate) and a perfect match between sample peak e′ and standard e (methyl *threo*-9,10-dichlorooctadecanoate). Column: DB-WAX.

presence of the *threo*-7,8 configuration of dichlorohexadecanoic acid was inferred by the chromatographic behavior of the positional isomers and rationalized by the known characteristics of the β-oxidative metabolism.

Acknowledgements

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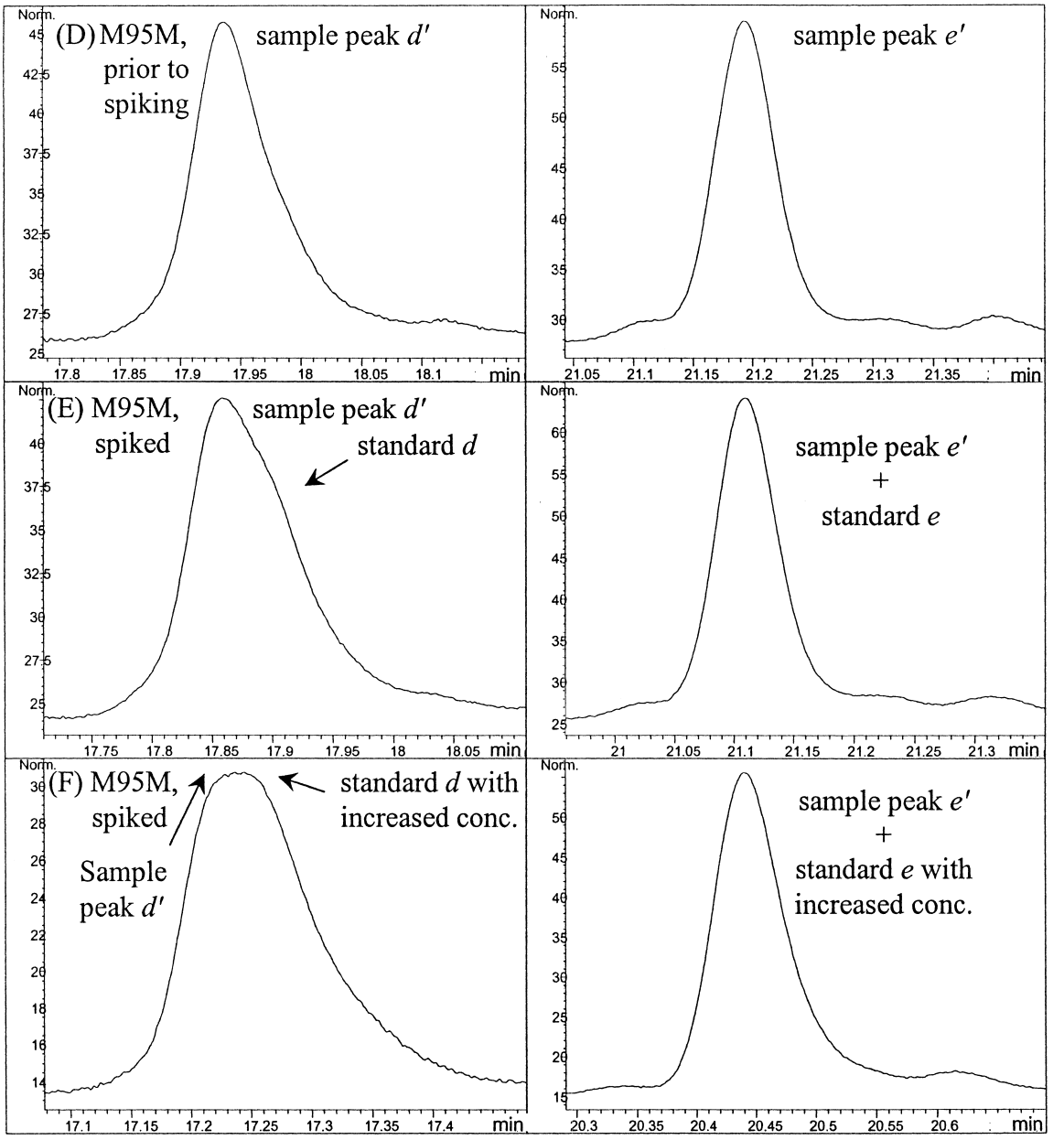

Fig. 12. (*continued*)

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