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# Enrichment of chlorinated fatty acids in fish lipids prior to analysis by capillary gas chromatography with electrolytic conductivity detection and mass spectrometry

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**Abstract**

Chlorinated carboxylic acids of fatty acid character have been shown to account for up to 90% of the extractable, organically bound chlorine (EOCl) in fish. To facilitate the detection of chlorinated fatty acid methyl esters (FAMEs) released from fish lipids, an enrichment was performed by removing the polyunsaturated FAMEs and saturated, straight-chain FAMEs through their complexation with silver ions and urea, respectively. The resulting about 30-fold increase in the concentration of chlorinated FAMEs allowed for their analysis, by gas chromatography (GC) with halogen-selective electrolytic conductivity detection, in lipids containing only 30 ppm (m/m) of EOCl. Following additional purification by thin-layer chromatography, methyl esters of dichlorotetradecanoic, dichlorohexadecanoic and dichlorooctadecanoic acids were indicated by GC–ammonia positive ion chemical ionisation mass spectrometry in a fish sample containing 1200 ppm (m/m) of EOCl.

*Keywords:* Fish; Environmental analysis; Silver ion complexation; Urea complexation; Pulp mill effluents; Fatty acid methyl esters, chlorinated; Chlorinated fatty acids; Lipids

## 1. Introduction

The occurrence of chlorinated organic compounds in fish from polluted waters is a matter of great concern. However, it may not have been generally conceived that the chlorine in chlorinated pollutants [such as polychlorinated biphenyls, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene (DDE) and chlorophenols] accounts for only 2–10% of the extractable, organically bound chlorine (EOCl) in fish [1–6]. By using gas chromatography (GC) with electrolytic conductivity detection (ELCD), we have found that a substantial part (70–90%) of EOCl in fish can be accounted for by chlorinated carboxylic acids [7,8], such as isomeric forms of dichloro- and tetrachloro-octadecanoic acids [9]. The lipids we studied held exceptionally high concentrations of EOCl (240 and 1200 ppm, m/m) and were obtained from fish caught in the receiving waters of pulp mills producing

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chlorine–chlorine dioxide-bleached kraft pulp, and chlorine-bleached sulphite pulp, respectively. It has been discussed whether chlorinated fatty acids generally occur in fish lipids [7,8,10–12], which commonly have a concentration of EOCl in the range of 30–60 ppm.

The GC–ELCD method has a detection limit of 50 pg of chlorine, corresponding to about 250 pg and 400 pg of di- and monochlorinated methyl octadecanoates, respectively [7]. On analysing fatty acid methyl esters (FAMEs) having a concentration of EOCl of about 30–60 ppm, chlorinated FAMEs cannot be detected, because their individual concentrations are below the ELCD detection limit. GC with mass spectrometric detection (GC–MS) in the selected ion monitoring mode can reach a detection limit for methyl dichlorooctadecanoate of about 100 pg [13]. GC–MS in the full scan mode, which is required in an unconditional search for chlorinated FAMEs, will result in a lower sensitivity. Thus, to detect chlorinated FAMEs the total load of FAMEs has to be increased, which, however, will overload the column and impair the column efficiency. A relative increase in the concentration of chlorinated FAMEs in relation to unchlorinated ones would therefore be desired.

In the present study, polyunsaturated FAMEs and straight-chain FAMEs were selectively removed from the transesterified sample by forming the corresponding silver ion complexes and urea inclusion complexes, respectively. Chlorinated FAMEs were thereby enriched, allowing a higher loading of chlorinated FAMEs on the capillary column instead of increasing the total load of FAMEs. The enrichment method was developed using FAMEs containing 1200 ppm of EOCl and thereafter applied to FAMEs containing lower concentrations of EOCl. Chlorinated FAMEs in the sample containing 1200 ppm of EOCl were further purified by silica gel thin-layer chromatography (TLC) and studied by MS with ammonia positive ion chemical ionisation (PICI).

## 2. Experimental

### 2.1. Solvents and reagents

Urea of purum grade was washed with cyclohexane and dried under nitrogen before use. All other chemicals used were of pro analysi grade. Doubly distilled water was rinsed with cyclohexane before use. All glassware was washed with acetone and heated at 550°C before use [14].

### 2.2. Reference compounds

Homologous series of FAMEs, separated by two methylene units (methyl hexanoate to methyl triacontanoate, methyl *cis*-9-hexadecenoate to methyl *cis*-15-tetracosenoate), and fish FAMEs of a known composition (Larodan Fine Chemicals, Malmö, Sweden) were used as retention standards for a tentative identification of the FAMEs indicated by the peaks in the flame ionisation detector (FID) chromatograms. The amounts of FAMEs in the fish samples were estimated by FID in relation to an internal standard, methyl nonadecanoate (Merck, Darmstadt, Germany). Methyl *threo*-9,10-dichlorooctadecanoate and methyl 9(10)-monochlorooctadecanoate (Synthelec, Lund, Sweden) were used as external standards for the ELCD.

Dichlorinated FAMEs (methyl *threo*-9,10-dichlorohexadecanoate, methyl *threo*-9,10-dichlorooctadecanoate, methyl *threo*-11,12-dichloroeicosanoate, methyl *threo*-13,14-dichlorodocosanoate, and methyl *threo*-15,16-dichlorotetracosanoate) were produced from the corresponding monounsaturated analogues according to Lyness and Quackenbush [15]. A series of monochlorinated FAMEs was produced from the monounsaturated FAMEs (methyl *cis*-9-hexadecenoate to methyl *cis*-15-tetracosenoate) according to Frøyen and Skramstad [16]. In order to account for possible losses, synthesised chlorinated FAMEs were subjected to the complexation procedures.

### 2.3. Fish lipids

Six fish samples obtained from different locations in Scandinavian coastal waters and one sample from the coast of Alaska were used in this study (Table 1). Eel (*Anguilla anguilla*) were obtained from the Ide fjord, between Sweden and Norway (eel A), and from the Oslo fjord (eel B), both of which were influenced by pulp mill effluents [5,7]. Flounder (*Platichthys flesus*) were obtained from the bight of Hanö in south Sweden, in the vicinity of a pulp mill (flounder A), and from an open, coastal area in the

Table 1
Conditions of exposure for the studied fish and concentrations of EOCl in the extracted fish lipids

| Fish species | Exposed to | EOCl (ppm) | Ref. |
|---|---|---|---|
| Eel A (*Anguilla anguilla*) | Chlorine-bleached sulphite mill effluent | 1200 | 5, 7 |
| Eel B (*A. anguilla*) | Chlorine–chlorine dioxide bleached kraft mill effluent | 57 | 5, 7 |
| Flounder A (*Platichthys flesus*) | Chlorine–chlorine dioxide bleached kraft mill effluent | 50 | 7 |
| Flounder B (*P. flesus*) | No known point source of chlorinated material | 30 | 7 |
| Atlantic salmon (*Salmo salar*) | Unknown[a] | 44 | 5 |
| Perch (*Perca fluviatilis*) | Chlorine–chlorine dioxide bleached kraft mill effluent | 40 | 8 |
| Pacific salmon (*Oncorhynchus nerka*) | No known point source of chlorinated material | n.d. | 17 |

[a] Several Swedish pulp mills are situated along the coast of the Bothnian Sea; n.d.=not determined.

south-west part of the bight, about 300 m off the shore (flounder B) [7]. Atlantic salmon (*Salmo salar*) were obtained from a coastal area of the Bothnian Sea [5], 40 km north of Härnösand (Sweden). Perch (*Perca fluviatilis*) were obtained in the Bothnian Sea, near the island of Kusön, Sweden, 10 km north of a pulp mill [8]. Pacific salmon (*Oncorhynchus nerka*) were sampled at the mouth of Copper River, Alaska [17].

Except for Pacific salmon, the lipids were extracted from the fish muscles using cyclohexane–isopropanol, 1:1 (v/v), and the crude extracts were washed with water and dried with sodium sulphate [7,18]. The concentrations of EOCl, determined by neutron activation analysis (NAA) (Institute for Energy Technology, Kjeller, Norway) [14], are given in Table 1. The lipids of Pacific salmon, extracted by using chloroform–methanol [19], were thoroughly evaporated to dryness at 40°C under a gentle flow of nitrogen and then dissolved in cyclohexane.

FAMEs were prepared from 0.1–1 g of lipids by acidic methanolysis using a mixture of cyclohexane and 0.8% (v/v) sulphuric acid in methanol [7]. FAMEs were similarly transesterified to fatty acid propyl esters using 1.5% sulphuric acid in *n*-propanol.

### 2.4. Silver ion complexation

Silver ion complexes of polyunsaturated FAMEs (Fig. 1A) were prepared following the procedure of Peers and Coxon [20]. FAMEs (100 mg) were dissolved in 2,2,4-trimethylpentane (4 ml) and the solution was vigorously shaken with an equal volume of ethanol–water, 1:1 (v/v), containing silver nitrate (1.0 g). The upper phase, mainly consisting of trimethylpentane and the uncomplexed FAMEs, was removed and dried over sodium sulphate.

The lower phase (ethanol–water) was diluted with water (4 ml) to dissociate the silver ion complexes. The polyunsaturated FAMEs, now released from the complexes, were extracted three times with 4 ml of *n*-hexane. The extract was dried as above.

The dry mass of the material recovered from the trimethylpentane and the ethanol–water phase, respectively, was determined by gravimetry after evaporating the solvents at 40°C under a gentle flow of nitrogen. The residues were dissolved in cyclohexane and studied by GC–ELCD–FID.

### 2.5. Urea complexation

Urea inclusion complexes of FAMEs (Fig. 1A) were formed according to Christie [21]. Urea (1.5 g) was moistened with methanol (0.15 g) and a solution of fish FAMEs (100 mg) in *n*-hexane (4 ml) was

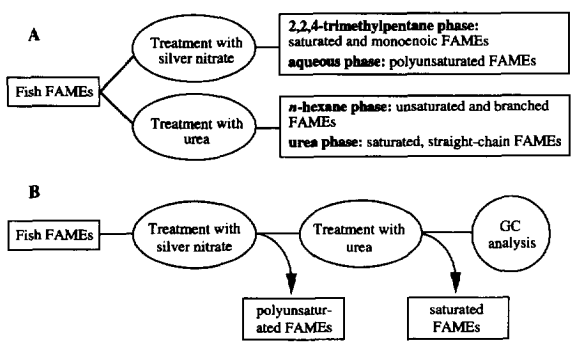


Fig. 1. Scheme for enrichment of chlorinated fatty acid methyl esters (FAMEs) by (A) complexation and removal of either polyunsaturated FAMEs or saturated straight-chain FAMEs, (B) consecutive complexation and removal of polyunsaturated FAMEs and saturated straight-chain FAMEs.

added. The slurry was vigorously shaken and left overnight at room temperature. The *n*-hexane phase, containing the uncomplexed FAMEs, was then removed after centrifugation. Additional, uncomplexed FAMEs remaining in the urea phase, were extracted three times with 3 ml of *n*-hexane. (If necessary, the urea was stirred with a clean glass rod to produce a well-mixed slurry of urea in *n*-hexane.) The *n*-hexane solutions were combined, washed twice with 5 ml of water and dried over sodium sulphate.

Water (10 ml) was added to the urea to dissociate the urea complexes. The straight-chain FAMEs released from the complexes were extracted three times with 3 ml of *n*-hexane. The combined extract was washed and dried, as above.

The dry mass of the material recovered in each of the two *n*-hexane solutions was determined by gravimetry after evaporating the solvent at 40°C under a gentle flow of nitrogen. The residues were dissolved in cyclohexane and studied by GC–ELCD–FID.

### 2.6. Enrichment of chlorinated FAMEs by consecutive removal of silver ion and urea complexes

FAMEs (100 mg) were first complexed with silver ions (Fig. 1B). The trimethylpentane phase (containing uncomplexed FAMEs) was separated and dried over sodium sulphate. After evaporating the solvent, the uncomplexed FAMEs were dissolved in *n*-hexane and subjected to urea complexation. The finally remaining, uncomplexed FAMEs were isolated as above, and studied by GC–ELCD–FID.

### 2.7. Thin-layer chromatography

Following the consecutive treatments with silver nitrate and urea, the remaining FAMEs of the eel lipid extract, originally containing 1200 ppm of EOCl, were further purified by silica gel TLC (No. 5547, Merck, Darmstadt, Germany). Following a prewash of the TLC plate with the elution medium, twenty spots, each containing 10 $\mu$g of FAMEs, were applied using a Camag TLC Spotter. Methyl *threo*-9,10-dichlorooctadecanoate was used as a reference. After elution with cyclohexane–diethyl ether (96:4, v/v), modified after Christie [22], the coating layers were scraped off in three fractions ($R_F$ intervals 0.14–0.18, 0.18–0.20, and 0.20–0.24). The lowest $R_F$ interval corresponded to that of methyl dichlorooctadecanoate. Diethyl ether (10+5 ml) was used to extract FAMEs from each of the collected fractions. The extracts were washed twice with 5 ml of water and dried as above. The FAMEs from different $R_F$ fractions were evaporated to dryness, dissolved in cyclohexane and studied by GC–ELCD–FID.

It proved necessary to use a low concentration of diethyl ether in the elution medium, else all FAMEs would be eluted within a narrow interval of a higher $R_F$ value.

### 2.8. Gas chromatography

GC analyses were carried out on a Varian Model 3700 gas chromatograph with two parallel detectors: a FID and an ELCD (Tracor/Varian, Model 1000) operated in the halogen-selective mode [7]. A glass splitter (Pressfit, Schmidlin Labor+Service, Switzerland) was used to split the column effluent between the two detectors. The samples were injected on-column [23] onto a fused-silica, capillary column (DB23, 50% cyanopropyl- and 50% methylpolysiloxane, J&W Scientific, Folsom, CA, USA, 30 m×0.32 mm I.D.; film thickness, 0.25 $\mu$m). Helium (99.9996%; Air Liquide Gas, Sweden) was used as carrier gas (4 ml/min). The injector temperature was 280°C, the FID temperature was 300°C and the ELCD base and reactor temperatures were 280°C and 850°C, respectively. The column temperature program was 90°C for 3 min, followed by 90–240°C at a rate of 4°C/min, and 20 min of isothermal conditions.

Linear retention indices ($I$) of monounsaturated, monochlorinated, and dichlorinated FAMEs, eluted within the linear temperature gradient of the GC oven program, were calculated in relation to saturated FAMEs, separated by two carbon atoms [24]. In the formula below, $t_R$ represents the retention time of an unchlorinated, saturated FAME of either $z$ or $z+2$ carbon atoms, respectively, and of the compound under study, $x$.

$$I = 100z + 200\frac{(t_R)_x - (t_R)_z}{(t_R)_{z+2} - (t_R)_z}$$

Table 2
Gravimetric recovery of dry mass in the different fractions isolated after complexation of eel FAMEs

| Complexation agent | Fraction | Recovery (%) | S.D. (%) ($n$=6) |
|---|---|---|---|
| Silver nitrate | Uncomplexed | 83.2 | 2.6 |
| | Complexed | 16.2 | 1.5 |
| | Total | 99.4 | |
| Urea | Uncomplexed | 17.6 | 2.4 |
| | Complexed | 76.2 | 2.1 |
| | Total | 93.8 | |
| Silver nitrate and urea | Uncomplexed | 5.5 | 0.8 |
| | Complexed | 78.0 | 5.7 |
| | Total | 83.5 | |

### *2.9. Mass spectrometry*

FAMEs were separated on a fused-silica capillary column (DB5, 5% phenyl- and 95% methylpolysiloxane, J&W Scientific, 25 m×0.25 mm I.D.; film thickness, 0.25 $\mu$m) fitted into a Hewlett-Packard Model 5890 GC in conjunction with a VG Model Trio-1S quadrupole MS, largely using the conditions for ammonia PICI-MS described by Sundin et al. [13]. The column temperature program was 90°C for 1 min, followed by 90–150°C at a rate of 12°C/min, followed by 150–260°C at a rate of 4°C/min, and 10 min of isothermal conditions. The electron energy was 70 eV and the ion source temperature was 120°C. The samples were analysed in the scan mode ($m/z$ 50–500, 1 scan/s).

A GC peak expected to represent cholesterol was investigated by GC–MS using electron impact (EI) ionisation. The conditions were as above, except for omitting the reaction gas.

## 3. Results and discussion

### *3.1. Enrichment of chlorinated FAMEs by removal of polyunsaturated FAMEs as their silver ion complexes*

The enrichment of polyunsaturated FAMEs in food samples, reported by Peers and Coxon [20], was obtained by selective extraction of the corresponding silver ion complexes from an organic solvent into water–ethanol. In the present study, we used this method to remove polyunsaturated FAMEs from the original FAME solution in order to enrich chlorinated FAMEs, assuming that chlorinated FAMEs in the fish sample, in conformity with saturated FAMEs, should not form silver ion complexes. To study the effect of silver ions on a chlorinated FAME, methyl nonadecanoate and methyl dichlorooctadecanoate were mixed and subjected to the silver nitrate treatment. Identical FID response ratios between the two compounds were obtained before and after the silver nitrate treatment, which indicates that the treatment with silver nitrate did not remove dichlorinated FAMEs.

About 16% of the original eel FAME dry mass was recovered from the silver ion complexes and 83% of the FAMEs remained uncomplexed (Table 2). Peers and Coxon [20], however, found that 26% of fish (mackerel) FAMEs formed silver ion complexes. The difference could possibly be explained by different proportions of polyunsaturated fatty acids in the two fish species.

GC–FID showed that 13% of the original FAMEs consisted of the polyunsaturated FAMEs: methyl eicosatetraenoate, methyl eicosapentaenoate, methyl docosapentaenoate and methyl docosahexaenoate. After the silver nitrate treatment, polyunsaturated FAMEs comprised 3% of the uncomplexed FAMEs, and 73% of the complexed FAMEs. Thus, the major portion of polyunsaturated FAMEs were removed by the silver ion complexation. A minor portion of methyl hexadecenoate and methyl octadecenoate was also found among the FAMEs that had formed complexes. This could be due to silver ions forming complexes also with monoenoic FAMEs [20], but to a lesser extent than with polyunsaturated FAMEs.

In comparison with the original FAMEs (Fig. 2A), the ELCD response of the uncomplexed FAMEs was

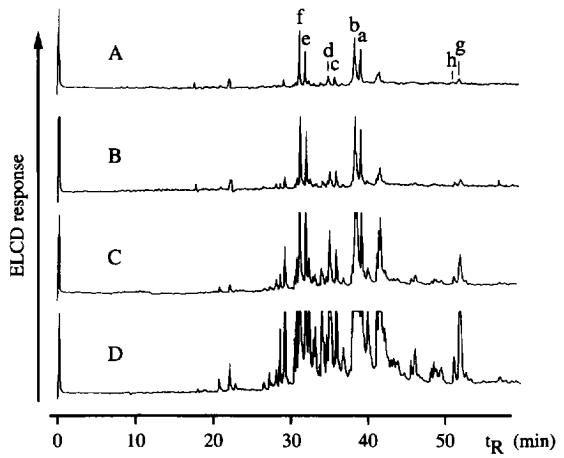


Fig. 2. ELCD gas chromatograms of chlorinated FAMEs liberated from eel lipids containing 1200 ppm of EOCl showing an increasing degree of enrichment: (A) original FAMEs; (B) uncomplexed FAMEs after the removal of polyunsaturated FAMEs as silver ion complexes; (C) uncomplexed FAMEs after removal of straight-chain FAMEs as urea complexes; (D) uncomplexed FAMEs after the removal of both silver ion complexes and urea complexes. The chromatograms were recorded at the same attenuation and show separations of 6 $\mu$g of dry mass in each run. Letters in A refer to the following chlorinated FAMEs, as deduced from linear retention indices and MS studies: a and b, *threo-* and *erythro*-forms of methyl 9,10-dichlorooctadecanoate [9]; c and d, *threo-* and *erythro*-forms of methyl 7,8-dichlorohexadecanoate; e and f, *threo-* and *erythro*-forms of methyl 5,6-dichlorotetradecanoate; g and h, two isomers of methyl tetrachlorooctadecanoate [9].

increased (Fig. 2B). No ELCD response was observed for the FAMEs that had formed silver ion complexes. Thus, an enrichment of chlorinated FAMEs was obtained by removal of polyunsaturated FAMEs as their silver ion complexes.

### *3.2. Enrichment of chlorinated FAMEs by removal of straight-chain FAMEs as their urea complexes*

Urea can form hexagonal prisms containing a channel in which straight-chain fatty acids and their esters can be included [25]. The relative amount of FAMEs that forms urea complexes depends on the mass ratio between urea and FAMEs [26] and the temperature [27]. The removal of unsubstituted, straight-chain FAMEs as their urea complexes can lead to an enrichment of bulky FAMEs, such as branched FAMEs, which cannot enter the urea channel [21,25]. Because the chlorine atoms in chlorinated FAMEs make the molecules occupy a larger space [28], we assumed that chlorinated FAMEs should behave similarly to branched FAMEs and utilised the urea complexation for removing unsubstituted, straight-chain FAMEs in the fish sample.

About 76% of the original eel FAME dry mass was recovered from the urea complexes and 18% of the FAMEs remained uncomplexed (Table 2). In agreement with previous data [21,25–27], the FAMEs that formed complexes were dominated by saturated and monoenoic FAMEs, such as methyl hexadecanoate, methyl hexadecenoate and methyl octadecenoate. The uncomplexed compounds were dominated by polyunsaturated FAMEs.

The ELCD response of the uncomplexed FAMEs following the treatment with urea (Fig. 2C) demonstrated that an enrichment of chlorinated FAMEs had occurred. Traces of the major, chlorinated compounds were found in the material that had formed complexes, possibly due to incomplete extraction of uncomplexed FAMEs from the urea.

To study if chlorinated FAMEs form urea complexes, mono- and dichlorinated FAMEs were added to the eel FAMEs and then subjected to the urea complexation. No additional dichlorinated FAMEs were found in the urea complexes. However, monochlorinated FAMEs extensively formed urea complexes (>90%), showing that one chlorine atom in the molecule does not constitute an obstacle to the formation of such complexes.

### *3.3. Enrichment of chlorinated FAMEs by consecutive removal of silver ion complexes and urea complexes*

Since chlorinated FAMEs in the eel sample neither formed complexes with silver ions nor with urea, both methods were suitable for the enrichment of chlorinated FAMEs. The recovery of uncomplexed material (dry mass) after the consecutive treatments with silver nitrate and urea was about 6% (Table 2). About 78% of the original FAMEs were recovered from the silver ion complexes and urea complexes. Sixteen per cent could not be accounted for.

The FID response of the uncomplexed FAMEs, which were dominated by monoenoic and dienoic species, corresponded to only 31–48% of the recovered dry mass (quantified in relation to the

internal standard). A late eluting compound, identified by GC–EI-MS as cholesterol, made up another 4–8% of the FID response. About 50% of the dry mass remaining after the complexations could not be accounted for by GC–FID. The discrepancy possibly could be explained by a concomitant enrichment of nonvolatile material in the sample. (Given that this explanation is correct, the nonvolatile material made up about 3% of the original sample, a concentration that easily would remain unnoticed.)

The removal of both polyunsaturated and saturated FAMEs by the consecutive silver ion and urea complexations resulted in an ELCD response (Fig. 2D) larger than could be estimated from silver nitrate treatment (Fig. 2B) and urea treatment alone (Fig. 2C). The consecutive treatments increased the ELCD response by a factor of 34 (standard deviation=7, $n=8$) in relation to the FAMEs quantified by using FID and the internal standard. The ELCD chromatograms indicated that no degradation of chlorinated compounds occurred. However, new compounds became detectable.

The concentrations of the two isomers of methyl tetrachlorooctadecanoate, previously identified among the eel FAMEs [9], were also increased in the uncomplexed FAMEs after removal of silver ion complexes and urea complexes (Fig. 2). This indicates that tetrachlorinated FAMEs neither formed complexes with silver ions nor with urea.

The concentration of EOCl (determined by NAA) in the original eel FAMEs was 1200 ppm [7], only 0.1% of which was accounted for by polychlorinated biphenyls and other well known organochlorine pollutants [5]. The ELCD response of the untreated FAMEs corresponded to 1300 ppm of chlorine (standard deviation=125, $n=4$). The amount of chlorine detected following the formation of silver ion and urea complexes was calculated to correspond to 1350 ppm based on the original amount of FAMEs (standard deviation=280, $n=8$). Thus, within the experimental error, similar results were obtained by NAA and GC–ELCD.

### 3.4. Purification of chlorinated FAMEs by TLC

Silica gel TLC was used to further purify the chlorinated FAMEs, because some unsaturated, unchlorinated FAMEs still dominated the uncomplexed material of the eel sample. The fraction characteristic of methyl dichlorooctadecanoate ($R_F$ interval 0.14–0.18) contained the major portion of chlorinated compounds (Fig. 3A). The $R_F$ interval 0.18–0.20 (Fig. 3B) contained small amounts of chlorinated compounds in addition to unchlorinated compounds. No chlorinated FAMEs were found in the $R_F$ interval 0.20–0.24 (Fig. 3C), which, however, contained the major portion of unchlorinated compounds. Fractions of other $R_F$ values were not studied.

The polar character of halogenated FAMEs has previously been used by Jones et al. [29] for the isolation of brominated FAMEs on aluminium oxide TLC plates. Similar to their results, we found, on separating untreated eel FAMEs by TLC, that polyunsaturated FAMEs dominated in the fraction containing the chlorinated FAMEs. It was therefore important to remove polyunsaturated FAMEs by

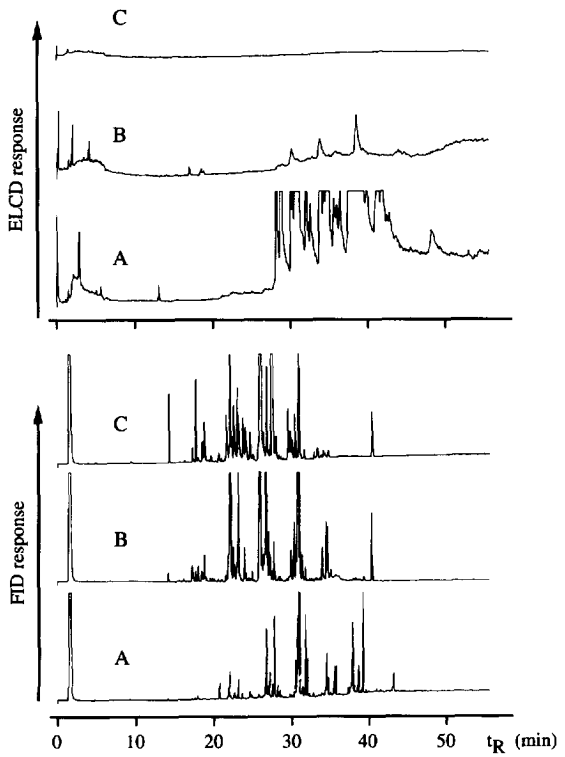


Fig. 3. ELCD and FID gas chromatograms obtained by separating the material of TLC fractions having $R_F$ intervals: (A) 0.14–0.18; (B) 0.18–0.20; and (C) 0.20–0.24. The chromatograms were recorded at the same attenuation and show separations of equal aliquots of the samples.

silver ion complexation before the TLC. It was also advantageous to perform urea complexation, because about 80% of the total dry mass of FAMEs was eliminated in this step, thus making it possible to apply to the TLC plate a material rich in chlorinated FAMEs.

### 3.5. Mass spectrometric identification of dichlorinated FAMEs

In accordance with earlier findings using ammonia PICI-MS [9,13], the reference compound methyl *threo*-9,10-dichlorooctadecanoate generated molecular mass related ammonium adduct chlorine isotope cluster ions ($[M+18]^+$) of $m/z$=384 and 386, and dechlorinated ammonium adduct ions ($[M-Cl_2+18]^+$) of $m/z$=314. Dechlorinated ions of $m/z$=315 and 316 were partly due to contribution of $^{13}C$ isotope.

Upon GC separation of eel FAMEs, isolated from the TLC plate in the $R_F$ interval 0.14–0.18, ions characteristic of methyl dichlorooctadecanoate (Fig. 4A) were found at two adjacent peaks. These compounds most likely were the diastereomers methyl *erythro*-9,10-dichlorooctadecanoate and methyl *threo*-9,10-dichlorooctadecanoate [9].

At the time of the MS study, no reference compounds were available with chain lengths shorter than that of methyl dichlorooctadecanoate. However, ammonium adduct ions of methyl dichlorohexadecanoate could be assumed to be represented by ions of $m/z$=356 and 358 and the dechlorinated moiety by ions of $m/z$=286. These values were obtained by subtraction of 28 mass units (two methylene groups) from the mass of the ammonium adduct of methyl dichlorooctadecanoate and the corresponding, dechlorinated product. Analogous ions of methyl dichlorotetradecanoate could be assumed to be of $m/z$=328, 330 and 258.

Ions were observed that indicated the presence of methyl dichlorohexadecanoate (Fig. 4B) and methyl dichlorotetradecanoate (Fig. 4C) in the purified eel FAMEs. The homologous nature of the dichlorinated FAMEs indicated by GC–MS, was further suggested by a linear relation between the number of carbon atoms in the fatty acid chains and the corresponding retention times on the DB5 column. The relationship was obtained within an interval of constantly increasing GC oven temperature (data not shown).

Although the magnitude of the ELCD peaks coinciding with the GC retention times of diastereomers of methyl *erythro,erythro*-9,10,12,13-tetrachlorooctadecanoate [9] increased following the consecutive treatments with silver nitrate and urea (Fig. 2), no tetrachlorinated compounds were found by GC–MS in the material purified by silica gel TLC. Jones et al. [29] reported that tetrabrominated FAMEs could be retarded to a larger extent than the dibrominated analogues, when employing aluminium

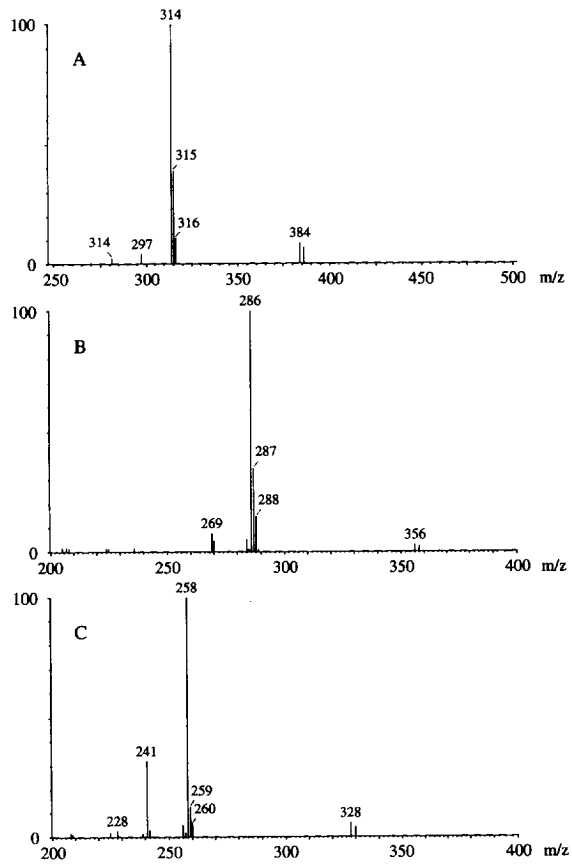


Fig. 4. Ammonia PICI mass spectra of: (A) methyl dichlorooctadecanoate, and compounds tentatively identified as: (B) methyl dichlorohexadecanoate and (C) methyl dichlorotetradecanoate, obtained on separation of eel FAMEs (eel A, originally containing 1200 ppm of EOCl) following consecutive formation and removal of silver ion complexes with polyunsaturated FAMEs and urea complexes with straight-chain FAMEs, and TLC.

oxide TLC for separation of brominated FAMEs from the naturally occurring FAMEs. Thus, a likely reason why no methyl tetrachlorooctadecanoate was found in the present GC–MS study is that the selected TLC interval was too narrow.

### 3.6. Tentative structures of the dichlorinated FAMEs

The *threo*-form of dichlorooctadecanoic acid most likely originated from chlorination of *cis*-9-octadecenoic acid. The isomer proposed to be the diastereomeric *erythro*-form, has been presumed to originate from chlorination of *trans*-9-octadecenoic acid formed by isomerisation of *cis*-9-octadecenoic acid in the acidic pulping process [9]. Both forms were found also in the current GC–ELCD (Fig. 2A) and GC–MS studies.

On the DB23 column, the GC retention time of methyl 9,10-*threo*-dichlorohexadecanoate, used as a reference compound, did not fully coincide with that of a compound assumed to represent methyl *threo*-dichlorohexadecanoate. Therefore, to correlate the mass spectrometric results to the GC–ELCD traces, linear retention indices were calculated for reference compounds and for four major, unknown peaks in the ELCD chromatograms. A straight line (Fig. 5) was obtained by plotting the retention indices of

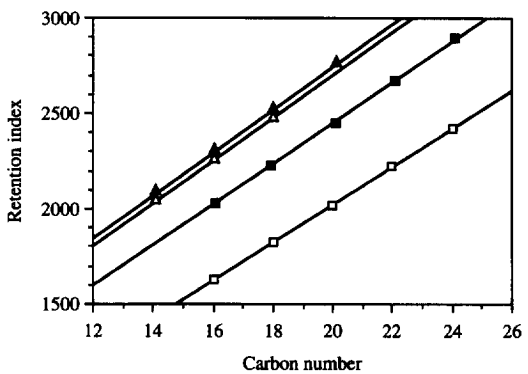


Fig. 5. Linear retention indices obtained on a DB23 column for: (□) monounsaturated FAMEs (reference compounds); (■) monochlorinated FAMEs (reference compounds); (△) dichlorinated FAMEs among the eel FAMEs (tentatively identified as the *erythro*-isomers); and (▲) dichlorinated FAMEs in *threo*-form ($C_{14}$, $C_{16}$ and $C_{18}$ tentatively being identified among the eel FAMEs; $C_{18}$ and $C_{20}$ were reference compounds). The correlation coefficients, $R^2$, were 1.000.

methyl *threo*-11,12-dichloroeicosanoate and methyl *threo*-9,10-dichlorooctadecanoate against their carbon chain lengths together with the retention indices for two ELCD peaks assumed to represent methyl dichlorohexadecanoate and dichlorotetradecanoate. Thus, these two peaks (Fig. 2A, peaks c and e) tentatively represented the homologues methyl *threo*-dichlorohexadecanoate and methyl *threo*-dichlorotetradecanoate.

An almost parallel line was found for another set of three ELCD detectable compounds (Fig. 5). Based on the earlier conclusion that one of these compounds is methyl *erythro*-9,10-dichlorooctadecanoate [9], the other two would be methyl *erythro*-dichlorohexadecanoate and methyl *erythro*-dichlorotetradecanoate (Fig. 2A, peaks d and f).

The high concentration of EOCl in eel from the Ide fjord most likely resulted from the discharges of effluents from a chlorine bleaching sulphite mill [5,7,9] and it is well known that 9,10-dichlorooctadecanoic acid can be formed by chlorination of 9-octadecenoic acid in pulp bleaching processes [30]. The ELCD peaks suggested to represent methyl dichlorotetradecanoate and methyl dichlorohexadecanoate gave considerable contributions to the total ELCD response (Fig. 2A). These compounds were formed either by chlorination in the pulp bleachery of hexadecenoic acid and tetradecenoic acid or by biodegradation of 9,10-dichlorooctadecanoic acid.

To our knowledge, no data on the occurrence of hexa- and tetradecenoic acids in sulphite mill effluents have been presented. In the total effluents of kraft paper mills [31] and in tall oil recovered from the black liquor after alkaline, kraft pulping processes [32], hexadecenoic acid has been found, albeit in low concentrations (1–4%) in relation to that of octadecenoic acid. However, halogenated hexadecanoic and tetradecanoic acids have been identified after exposing rats to esters of dichlorooctadecanoic acid and dibromooctadecanoic acid [29,33–35]. Jones et al. [29] showed that the bromine atoms in the dibromohexadecanoic and dibromotetradecanoic acids had the positions 7,8 and 5,6, respectively, implying that these compounds were $\beta$-oxidation products of 9,10-dibromooctadecanoic acid.

Therefore, the relatively high proportions of dichlorohexadecanoic and dichlorotetradecanoic acids

in the eel lipids suggest that these compounds were formed by β-oxidation of dichlorooctadecanoic acid in the fish tissues or in organisms preyed upon by the fish. Accordingly, the chlorine atoms in dichlorohexadecanoic and dichlorotetradecanoic acids indicated in the eel lipids should be in a 7,8-position and a 5,6-position, respectively.

### 3.7. Chlorinated fatty acids in fish lipids having low concentration of EOCl

Following the consecutive treatments of fish FAMEs with silver nitrate and urea, halogenated compounds could be detected with GC–ELCD also in fish samples having low concentrations of EOCl (Fig. 6A–E). To study the character of these halogenated compounds, the FAMEs were transesterified to propyl esters. Similar ELCD chromatograms, but with increased retention times, were obtained (a typical chromatogram is given in Fig. 6G). Because the increments corresponded to those obtained for chlorinated fatty acid propyl esters (FAPEs) of the eel A sample (detected by GC–ELCD) and to those of the normal, unchlorinated FAPEs (detected by GC–FID), the transesterification confirmed that the halogenated compounds were of carboxylic acid character.

Judging from the GC retention times, the major halogenated compound found in the fish samples shown in Fig. 6 (A–E) was methyl *threo*-5,6-dichlorotetradecanoate. The tentatively identified methyl *erythro*-5,6-dichlorotetradecanoate was found in eel A, eel B, flounder A, and perch, all of which were more or less directly exposed to the effluents of different pulp mills (Table 1). As the salmon is a non-stationary fish that moves over large areas, the fish rather represents the entire Baltic than the actual catch site [5]. Thus, only traces of methyl *erythro*-dichlorotetradecanoate were found in the salmon, even though it was obtained from the Bothnian Sea, where several pulp mills discharge their effluents. The presence of elevated concentrations of *erythro*-dichlorotetradecanoic acid in fish lipids might indicate a contamination by effluents from pulp bleacheries as a result of β-oxidation of *erythro*-9,10-dichlorooctadecanoic acid.

Pacific salmon from Alaska also contained halogenated FAMEs (Fig. 6F), showing that the presence of such compounds is not restricted to fish from Scandinavian coastal waters. The retention time of the major peak was close to that of methyl *threo*-dichlorotetradecanoate.

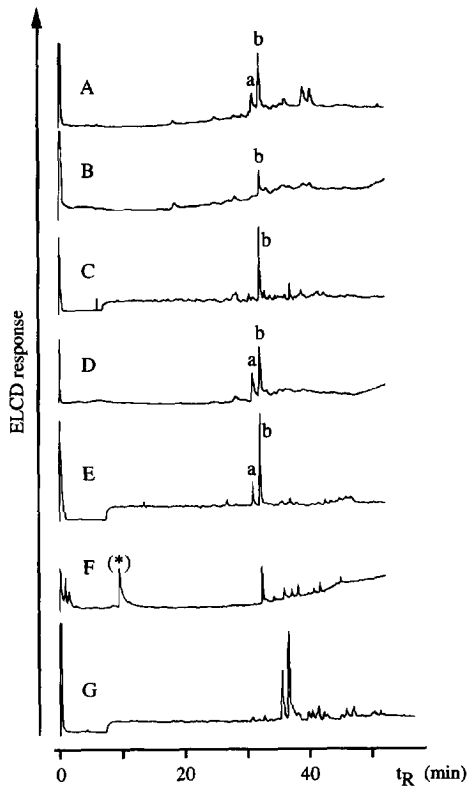


Fig. 6. ELCD gas chromatograms of FAMEs liberated from fish lipids and remaining after the consecutive formation and removal of silver ion complexes with polyunsaturated FAMEs and urea complexes with saturated, straight-chain FAMEs. (A) Flounder A, (B) flounder B, (C) Atlantic salmon, (D) perch, (E) eel B, and (F) Pacific salmon, (G) fatty acid propyl esters corresponding to the FAMEs in chromatogram E. The peaks a and b in chromatograms A to E have retention times coinciding with those of the *erythro*- and *threo*-forms of methyl 5,6-dichlorotetradecanoate, respectively. (*) represents an artifact obtained on shutting the detector vent.

Using external standards, we estimated that 35–75% of the EOCl of the original eel B lipids was accounted for by GC–ELCD. In the other fish samples, 20–60% of EOCl was detected (flounder A 32–42%, flounder B 57%, salmon 20% and perch 25–30%). In a previous study of the flounder A

lipids [7], EOCl was determined in fractions containing non-acidic (neutral) and acidic compounds following hydrolysis or transesterification reactions. We then estimated that chlorinated carboxylic acids accounted for 65–75% of EOCl. The lower recoveries found in the current study possibly suggest that several chlorinated fatty acids were present in concentrations below the detection limit of the method.

## 4. Conclusions

This study supports earlier findings that ester-bound, chlorinated fatty acids are dominating contributors to EOCl in fish tissues.

Dichlorinated and tetrachlorinated FAMEs in fish samples can be enriched by selective removal of polyunsaturated and saturated, straight-chain FAMEs through the complexation with silver ions and urea, respectively.

Silica gel TLC can be used for an additional purification of the chlorinated FAMEs, provided that polyunsaturated FAMEs are first removed.

The presence of elevated concentrations of *erythro*-dichlorotetradecanoic acid in fish lipids might indicate a contamination by effluents from pulp bleacheries.

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