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Identification of chlorinated fatty acids in fish lipids by partitioning studies and by gas chromatography with Hall electrolytic conductivity detection

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ABSTRACT

Chlorinated compounds in fish lipids [determined by neutron activation analysis as extractable, organically bound chlorine (EOCl)] were characterized by liquid–liquid extractions after enzymatic hydrolysis and after forming fatty acid methyl esters (FAMEs). Most of the chlorinated compounds in lipids from four different fish samples could be hydrolysed. Comparison with results of methanolysis of two of the fish lipids indicated that chlorinated fatty acids made up the major portion of EOCl. Using gas chromatography (GC) with electrolytic conductivity detection (ELCD), chlorinated compounds were found among FAMEs of eel lipids containing 1200 ppm of EOCl. Approximately 90% of EOCl was detected by GC–ELCD. The GC-detectable compounds are suggested to be methyl esters of chlorinated fatty acids and 9,10-dichlorostearic acid was tentatively identified after co-injection of the synthesized compound.

INTRODUCTION

Much work has been devoted to the identification of organochlorine compounds in fish. Organochlorine pollutants, such as polychlorinated biphenyls (PCBs), DDT and DDE, in fish tissues are normally traced by gas chromatography (GC) with electron-capture detection (ECD) or mass spectrometric (MS) detection. The chlorinated pollutants are first isolated in a crude extract, which also contains the fish lipids. After thorough purification and removal of lipid components, the sample can be subjected to GC and the amount of chlorine connected with the identified substances can be calculated. The total content of chlorine in hydrophobic compounds can be measured by neutron activation analysis (NAA) after elimination of chloride from the lipid-containing extract [1–3]. This measure expresses the extractable, organically bound chlorine (EOCl) and assesses the upper limit of chlorine that is to be accounted for by other techniques. By comparing the results obtained with GC and NAA, it has been found that compounds identified by GC–ECD or GC–MS can account for 0.1–16% of the EOCl in fish tissues [4–6] and 1–8% of the EOCl in sediments [5,7]. The major portion of EOCl in fish and

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sediments is of relatively high molecular weight [2,8,9], and part of the chlorinated material in fish lipids can be hydrolyzed, resulting in the release of acidic compounds from neutral (non-acidic) material [10]. These findings suggest that the unidentified organochlorine substances in fish lipids to a large extent are chemically bound in acylglycerols or similar esters and that the acidic hydrolysis products are either chlorinated carboxylic acids or chlorinated phenols.

As ECD has been reported to have a low sensitivity for methyl esters of chlorinated stearic acids [7,11], it is of limited value for studies of chlorinated lipid constituents such as chlorinated fatty acids. In addition to ECD, other halogen-sensitive GC detection methods have been developed. Karmen [12] coupled two flame ionization detectors in series, and studied the ionization due to metal chlorides in the upper flame. The detection limit was in the range of 1 ng and could not compete with ECD. Atomic emission detection (AED) and electrolytic conductivity detection (ELCD) are halogen-selective detection methods which are commercially available. AED can identify individual halogens and the response is based on their spectral emission produced by the high temperature in a plasma. Although the principle has been known for a long time, it is only lately that AED has become commercially available. ELCD was first developed by Piringer and Pascalau [13] for the determination of carbon. Coulson [14] used a similar system for the selective detection of compounds containing either halogens, nitrogen or sulphur. In this type of detector, the GC eluate is pyrolyzed. The reaction products are transported to a mixing chamber, mixed with a suitable solvent and the conducting properties of the solvent are registered. The Coulson detector has been further improved by Hall [15] and, in a recent configuration, the detection limit using ELCD was lowered to the picogram range. The US Environmental Protection Agency has recommended ELCD for the detection of PCBs and other halogenated substances in certain environmental samples.

In this study, fish lipids were subjected to enzymatic hydrolysis and methanolysis. The products were partitioned by liquid–liquid extraction at different pH and NAA was used to measure the chlorine in fractions containing acidic and neutral (non-acidic) compounds. The chlorine in chlorinated carboxylic acids was then determined. Fatty acid methyl esters (FAMEs) were detected by GC–ELCD and by GC-flame ionization detection (FID), used in parallel. Methyl esters of chlorinated stearic acids were used as external standards for calibrating the ELCD response and the total ELCD response was compared with the amounts of organically bound chlorine that were injected. The ECD response was also studied for some samples containing FAMEs.

EXPERIMENTAL

Preparation of fish lipids and EOCl determination

Eels (*Anguilla anguilla*) were obtained from the receiving waters of two Norwegian pulp mills producing bleached pulp, as described in Håkansson *et al.* [6]. One eel sample, A (39 fish), was obtained from the vicinity of a magnesium acid sulphite mill (chlorine bleaching) in the narrow fiord Idefjord, between Sweden and Norway. The other eel sample, B (sixteen fish), was obtained from the receiving waters of a kraft mill (chlorine dioxide/chlorine bleaching), discharging to a coastal area of the Oslo fiord (after determining EOCl in this sample, it served as a reference to eel A in the GC analysis). Flounders (*Platichtys flesus*) were caught in the bight of Hanö in southern Sweden. Samples C and D (three flounders each) were obtained from the receiving waters of a kraft mill, using chlorine/chlorine dioxide bleaching after an oxygen delignification stage. Sample E (five flounders) was obtained from an open, coastal area in the bight of Hanö, 10 km north of Simrishamn, about 300 m offshore, at a distance of 45 km from any point source of chlorinated material.

The determination of EOCl included homogenization of the fish fillets, extraction with cyclohexane–2-propanol (1:1), removal of 2-propanol and inorganic chlorine by subsequent washings with distilled water (pH 2, H_2SO_4) and drying the cyclohexane phase over anhydrous Na_2SO_4 [1,2]. The concentration of EOCl in the extract was determined by NAA according to Gether *et al.* [1]. The relative standard deviation of the method is about 10% [2,16].

Enzymatic hydrolysis and partitioning of lipids

Enzymatic hydrolysis of fish lipids, dissolved in

cyclohexane, was performed for 20 h using an immobilized *Mucor miehei* lipase (Lipozyme IM 20, activity 64 BIU/g) (Novo Industry, Copenhagen, Denmark) and distilled water [10].

The partitioning of hydrolysed and unhydrolysed lipids into neutral (non-acidic) and acidic substances was effected by liquid–liquid extractions between cyclohexane and aqueous buffers at different pH (Fig. 1). 2-Propanol was added to break emulsions [10]. After partitioning, the dry mass of the lipid residues that could be extracted with cyclohexane was determined gravimetrically after evaporating the solvent under nitrogen at 30°C. Each residue was dissolved in a small volume of cyclohexane and the chlorine content was determined by NAA.

Esterification and partitioning of lipids

Two methods were used for esterification of the unhydrolysed lipids to FAMEs, following Christie [17]. The methods were slightly modified by merely selecting solvents that were known to maintain low concentrations of EOCl in blank procedures. In one method, eel lipids were subjected to alkaline transesterification. Four portions of eel lipid, *ca.* 50 mg each, were each dissolved in 1 ml of cyclohexane, mixed with a solution of 0.5 *M* Na in methanol (2 ml) and heated at 50°C for 10 min. Water (3 ml) was added and the FAMEs in each sample were extracted three times with 3 ml of cyclohexane [17]. The FAME solutions were transferred to a separation funnel.

The aqueous phases were combined to one sample, which was acidified with H_2SO_4 from a pH of about 12 to 2 (Fig. 1), and shaken three times with 12 ml of cyclohexane. The cyclohexane solutions, containing uncharged, acidic substances (possibly chlorophenolic compounds and free fatty acids), were transferred into another separation funnel.

The two extracts were rinsed twice with water (pH 2), dried, concentrated by evaporation to small volumes and analysed for chlorine by NAA. The dry masses were determined as described above.

The alkaline transesterification was also performed with one sample containing 212 mg of eel lipid, the procedure being the same but on a four times larger scale.

In the other method, flounder lipids were subjected to acidic methanolysis. Six portions of flounder lipid, about 25 mg each, were each dissolved in 1 ml of cyclohexane, mixed with a solution of 1.5% (w/v) H_2SO_4 in methanol (2 ml), and heated at 60°C for 12 h [17]. The solutions were combined into one sample. Lipid residues in the small tubes were washed out by two additions of cyclohexane and transferred to the combined sample (a total cyclohexane volume of 30 ml). Acidic substances were removed by shaking the extract against a pH 9 buffer (50 ml). After centrifugation, the cyclohexane, containing FAMEs, was transferred into a separation funnel. The extraction of FAMEs was repeated once with another portion of cyclohexane (24 ml).

After acidifying the buffer to pH 2, uncharged, acidic substances were twice extracted from the water phase with cyclohexane (25 + 25 ml).

The two extracts were rinsed with water, dried, analysed for chlorine and the dry masses determined as above.

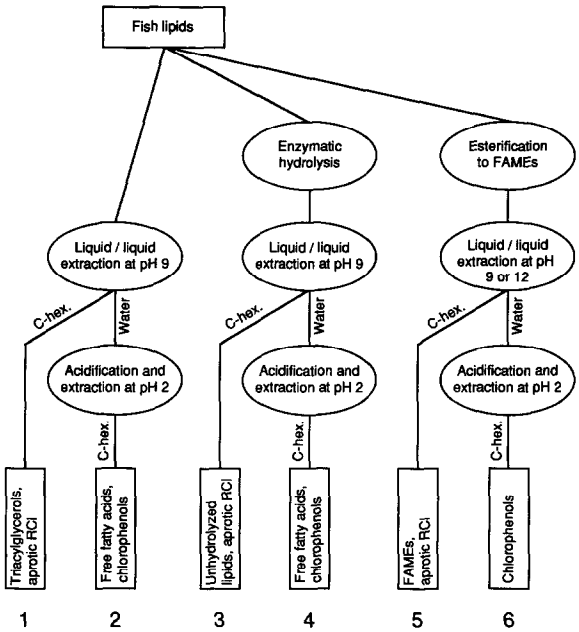

Fig. 1. Partitioning scheme used for characterizing chlorinated organic substances (RCl) in untreated fish lipids, lipids hydrolysed with lipase and esterified lipids. The partitioning of triolein and oleic acid was studied by silica gel thin-layer chromatography according to Håkansson *et al.* [6]. FAMEs were determined by GC–FID. PCBs in hydrolysed samples were determined by GC–ECD after treatment with concentrated sulphuric acid. Free chlorophenols (added as reference substances) were determined previously [10] by reversed-phase liquid chromatography. The numbers 1–6 at the bottom are used in Tables I and II.

Free fatty acids, obtained by enzymatic hydrolysis of the eel lipids A, were converted into FAMEs by acidic esterification, using 2 *M* HCl in methanol (60°C, 30 min [17]). Water was added and the FAMEs were extracted with cyclohexane.

GC conditions

The samples that were obtained after the esterification procedures were dissolved in cyclohexane and the FAMEs were studied by GC (Varian 3500) with FID and ELCD, used in parallel. A glass splitter (Pressfit, Schmidlin Labor + Service) divided the column flow to the two detectors with a splitting ratio close to 1:1. In part of the study, the electrolytic conductivity detector was replaced with an electron-capture detector.

The samples were injected on-column into a NB-54 fused-silica capillary column (18 m × 0.32 mm I.D., film thickness 0.25 μm) (HNU-Nordion). Helium was used as the carrier gas (4 ml/min). After a delay of 1 min, the injector was programmed from 90 to 280°C at a rate of 200°C/min. The column temperature was programmed from 90°C (held for 3 min) to 280°C at 6°C/min.

The ELCD instrument (Tracor/Varian, Model 1000) was mounted above a hole drilled in the column oven ceiling, close to the FID instrument. It was operated at a base temperature of 280°C and a temperature of 830°C in the nickel reactor tube. Helium (99.995%) was used as make-up gas (26 ml/min) and hydrogen (99.9995%) as reaction gas (23 ml/min). The detector was bypassed by vent during the first 2 min, which prevented the solvent from reaching the reactor tube. A PTFE tube transferred the pyrolysis products from the reactor tube to the electrolytic conductivity cell, through which 1-propanol was passed at a flow-rate of 0.53 ml/min. Before entering the cell, the 1-propanol was pumped through an ion-exchange resin (halogen form) which removed halogen ions. When used in the halogen-selective mode, other solutes containing nitrogen or sulphur do not interfere in ELCD. The ion-exchange resin and solvent were supplied in assembly by Varian. Any 1-propanol that evaporated was replaced with new solvent (>99.8%, Aristar) (BDH).

The FID and ECD systems were operated at 320°C and nitrogen was used as make-up gas (20 ml/min in each detector).

A series of homologous FAMEs (FA mix ME 62, Larodan) and a fish fat (methyl esters) of known constituency (Qualmix fish, Larodan) were used for the tentative identification of the FID peaks in the sample chromatograms. The FAMEs in the samples were then determined in relation to methyl nonadecanoate (internal standard) and the amounts of FAMEs were compared with the gravimetrically determined dry masses.

Methyl esters of 9,10-dichlorostearic acid and a mixture of 9- and 10-monochlorostearic acid (Synthelec, Lund, Sweden) were prepared by acidic esterification. The identity of these chlorinated standards has been verified by GC–MS [11]. These substances were used as external standards for ELCD. A PCB mixture (Clophen A 50, Bayer) was also used for reference purposes.

RESULTS AND DISCUSSION

Concentrations of halogenated compounds in fish

The eels, caught in the vicinity of the sulphite mill, contained about 1200 μg of EOCl per gram of lipid. Eels from the receiving waters of the Norwegian kraft mill and the flounders caught in the vicinity of the Swedish kraft mill had EOCl concentrations of 30–60 μg/g lipid. Flounders from the low-impact area of Simrishamn had an EOCl concentration of slightly less than 30 μg/g lipid. It can be argued that the reported data cannot be used directly for comparing the effects on the environment of the two pulp production methods because the Idefjord fiord, into which the sulphite mill discharged its effluent, is narrow and has a low water exchange rate. However, high EOCl concentrations (200–2000 μg/g lipid) have been found in freshwater fish close to a discharge of bleach effluents from an old calcium-based sulphite mill [18]. Additionally, sulphite mills producing bleached pulp can be expected to have a greater impact on the EOCl concentrations in fish in the receiving waters than can be expected for kraft mills, because EOCl in bleach liquors from sulphite mills and from kraft mills makes up about 3–15% and 0.5–2%, respectively, of the total content of organically bound halogens in the liquors [19–21]. Bleaching of pulp, produced according to the sulphite process, can therefore probably produce more organochlorine compounds that can bioaccumulate than the bleaching of kraft pulp.

TABLE I

PARTITIONING OF LIPIDS, EOCl AND EOBr IN NEUTRAL AND ACIDIC SUBSTANCES BEFORE AND AFTER AN ENZYMATIC HYDROLYSIS

The EOCl blank values are substracted and include all solvents and (when used) enzyme.

Sample	Original lipid extract			Fraction[a]	Recovered lipid		Recovered EOCl		Recovered EOBr		EOCl blank (μg)
	Lipid (g)	EOCl (μg)	EOBr (μg)		g	%	μg	%	μg	%	
Undrolysed eel lipid (A)	1.003	1220	2.6	1 (neutral)	0.932	93	1200	98	n.a.	–	3.8
(EOCl: 1220 μg/g fat)				2 (acidic)	0.005	1	25	2	n.a.	–	0.6
Hydrolysed eel lipid (A)	1.002	1220	2.6	3 (neutral)	0.205	20	190	16	n.a.	–	1.2
				4 (acidic)	0.546	55	530	43	n.a.	–	1.1
Hydrolysed eel lipid (A)	1.009	1230	2.6	3 (neutral)	0.211	21	210	17	n.a.	–	1.2
				4 (acidic)	0.562	56	560	46	n.a.	–	1.1
Unhydrolysed flounder lipid (C)	0.346	16.6	n.a.[b]	1 (neutral)	0.280	81	13	78	n.a.	–	0.3
(EOCl: 48 μg/g fat)				2 (acidic)	0.055	16	0.5	3	n.a.	–	0.4
Hydrolysed flounder lipid (C)	0.356	17.1	n.a.	3 (neutral)	0.068	19	3.1	18	n.a.	–	0.6
				4 (acidic)	0.224	63	4.3	25	n.a.	–	0.2
Hydrolysed flounder lipid (C)	0.366	17.6	n.a.	3 (neutral)	0.070	19	3.0	17	n.a.	–	0.6
				4 (acidic)	0.238	65	6.3	36	n.a.	–	0.2
Unhydrolysed flounder lipid (D)	0.347	10.4	n.a.	1 (neutral)	0.275	79	11	106	n.a.	–	0.3
(EOCl: 30 μg/g fat)				2 (acidic)	0.062	18	1.7	16	n.a.	–	0.4
Hydrolysed flounder lipid (D)	0.409	12.3	n.a.	3 (neutral)	0.070	17	3.3	27	n.a.	–	0.6
				4 (acidic)	0.276	67	3.2	26	n.a.	–	0.2
Hydrolysed flounder lipid (D)	0.348	10.4	n.a.	3 (neutral)	0.064	18	2.9	28	n.a.	–	0.6
				4 (acidic)	0.235	68	2.3	22	n.a.	–	0.2
Unhydrolysed flounder lipid (E)	0.299	8.4	1.1	1 (neutral)	0.295	99	7.6	91	1.1	104	0.2[d]
(EOCl: 28 μg/g fat)				2 (acidic)	n.d.[c]	–	n.d.	–	n.d.	–	0.2[d]
Hydrolysed flounder lipid (E)	0.316	8.8	1.2	3 (neutral)	0.031	10	1.7	19	0.2	16	0.1[d]
				4 (acidic)	0.228	72	2.7	31	0.2	16	0.2[d]

[a] The numbers 1–4 characterizing the fractions refer to Fig. 1.
[b] n.a. = Not analysed.
[c] n.d. = Not detected.
[d] EOBr blanks were 0.08 μg.

Characterization of chlorinated compounds and lipids

Untreated lipids. Partitioning with liquid–liquid extractions showed that most lipids and chlorinated compounds in the untreated samples of eel A and flounder E were of a neutral (non-acidic) character (Table I). This result agreed with that for a previously performed characterization of EOCl in cod liver lipids [10]. However, some hydrolysis (lipolysis) had occurred with the flounder samples C and D during storing the fish at −20°C for 15 months before homogenization and extraction. This led to 16–18% of the lipid dry mass and part of the chlorinated substances to be recovered as acidic material. The lipolysis found with the two flounder samples is a phenomenon well known by lipid chemists, but is usually disregarded when persistent, organochlorine pollutants are studied.

Enzymatic hydrolysis of lipids. After the enzymatic hydrolysis, neutral material made up 10–20% of the supplied lipids (Table I). The enzymatic hydrolysis also reduced the proportion of chlorinated compounds of neutral character (to 15–30% of the supplied EOCl) and part of the chlorinated material (22–45%) was recovered as acidic compounds. The neutral lipids, remaining after the enzymatic hydrolysis, were again treated with lipase but no further hydrolysis could be detected. The extent of hydrolysis was therefore not limited by the amount of water added.

Each experimental set-up for testing the partitioning of chlorinated compounds was accompanied by a blank sample. In spite of this, the recovered EOCl for one flounder sample exceeded 100%, showing the difficulties in handling small amounts of EOCl in the laboratory.

The partitioning studies showed that most of the chlorinated compounds in fish, exposed to pulp bleach liquors, did not consist of aprotic compounds, otherwise they should be recovered as neutral material after the enzymatic hydrolysis. Further, there was no evident difference in the hydrolysable properties of the chlorinated compounds in fish from the receiving waters of the two pulp mills and fish from the area of Simrishamn with a low anthropogenic impact. The main result of this study agrees with an earlier observation [10] that most chlorinated compounds in marine fish can be hydrolysed by lipase, and the result was independent of the EOCl levels and catch localities. The observation also corresponds to that obtained for brominated compounds [characterized with NAA as extractable, organically bound bromine (EOBr)] in marine fish [22,23] (Table I). It can therefore be concluded that halogenated compounds make up part of the fish lipids.

Alkaline transesterification of eel lipids. The lipids that were extracted from eel caught in the receiving waters of a sulphite mill (eel A) were converted into FAMEs. On performing the alkaline transesterification in one batch, the dry mass in the FAME fraction corresponded to slightly more than 90% of the supplied eel lipids (Table II). This corresponds reasonably well with the theoretical recovery from a triacylglycerol. A lower recovery was obtained when the eel lipids were transesterified in several small portions. However, it was necessary to establish the percentage of the neutral material that was actually in the form of FAMEs because a triacylglycerol and a FAME would be partitioned similarly. Comparisons with the results of GC–FID showed that FAMEs made up 95–102% of the dry mass of the neutral material, *i.e.*, the esterification yield (Table II), showing that the transesterification was complete. The finding also indicates that lipids extracted according to the EOCl method are dominated by triacylglycerols, as glycerophosphatides carry only two fatty acids in each molecule. Håkansson *et al.* [6] drew the same conclusion after separating similar fish lipids by silica gel chromatography, finding that 93–97% of the untreated lipids was eluted in a fraction characterized by triacylglycerols.

Acidic esterification of flounder lipids. The partitioning of the unhydrolysed flounder sample C showed that acidic compounds made up about 17% of the lipid dry mass (Table I). The esterification to FAMEs was therefore performed under acidic conditions because an alkaline transesterification cannot transform free fatty acids into their methyl esters. After the acidic esterification, which can form FAMEs from both acylglycerols and free fatty acids, 105–106% of the supplied lipids were recovered as neutral substances (Table II). The recovery of > 100% was probably due to the fact that part of the lipids were free fatty acids, which on acidic esterification became the corresponding methyl esters, thereby increasing the weight. By correlating the FAMEs determined by GC–FID with the dry

TABLE II

PARTITIONING OF LIPIDS AND EOCl IN NEUTRAL AND ACIDIC SUBSTANCES AFTER TRANSESTERIFICATION

The EOCl blank values are subtracted.

Sample	Original lipid extract		Fraction[a]	Recovered lipid		Recovered EOCl		EOCl blank (μg)	Esterification yield (%)
	Lipid (g)	EOCl (μg)		g	%	μg	%		
Esterified eel fat (A)	0.212[b]	260[b]	5 (neutral)	0.193	91	265	102	7.5	95
(EOCl: 1220 μg/g fat)			6 (acidic)	0.003	1	8	3	6.0	
Esterified eel fat (A)	0.211[c]	260[c]	5 (neutral)	0.157	74	170	65	12.2	102
			6 (acidic)	0.011	5	40	15	5.0	
Esterified flounder fat (C)	0.155[d]	7.5[d]	5 (neutral)	0.164	106	6.7	90	0.1	98
(EOCl: 48 μg/g fat)			6 (acidic)	0.003	2	n.d.[e]	–	0.0	
Esterified flounder fat (C)	0.149[d]	7.2[d]	5 (neutral)	0.156	105	5.9	82	0.1	95
			6 (acidic)	0.003	2	0.1	1	0.0	

[a] The numbers 5 and 6 characterizing the fractions refer to Fig. 1.
[b] One sample.
[c] Four samples of *ca.* 50 mg.
[d] Six samples of *ca.* 25 mg.
[e] n.d. = Not detected.

mass of the samples, the esterification yield was calculated to be 95–98%. The acidic esterification of flounder lipids therefore proceeded similarly to the alkaline transesterification of eel lipids and the esterification experiments could be used for discussions about EOCl as part of the fish lipids.

Contribution to EOCl from chlorinated carboxylic acids. The hydrolysis experiments alone could not ascertain whether the chlorine-containing esters were chlorinated phenols esterified with fatty acids or acylglycerols containing chlorinated carboxylic acids. However, after forming FAMEs from acylglycerols by transesterification (alkaline and acidic) and from free fatty acids by acidic esterification, there was a close relationship between the dry masses and the amounts of EOCl in fractions containing FAMEs and acidic substances, respectively (Table II). As repeated hydrolysis did not increase the yield of hydrolysis products, the chlorinated material remaining in the neutral fraction after hydrolysis was regarded as aprotic substances. The chlorine associated with carboxylic acids was therefore calculated as the difference between the percentage of EOCl in the neutral fraction containing FAMEs (Table II) and the percentage of EOCl in the neutral fraction after the enzymatic hydrolysis (Table I). Using this approach, chlorine in chlorinated carboxylic acids was calculated to account for 50–85% of EOCl in eels exposed to chlorinated compounds from the sulphite mill and 65–75% of the EOCl in flounders caught in the receiving waters of a kraft mill.

The reasoning above assumes that any fatty acids, esterified with chlorophenols, were transesterified to methyl esters in the esterification procedures, thereby releasing free chlorophenols. If this should not hold, part of the EOCl recovered in the neutral fraction might actually have been fatty acid esters of chlorophenols. However, transesterification is a reversible reaction and the position of the equilibrium depends on the relationship between the concentrations of the alcohol added and the ester. The large excess of methanol thus favours the transesterification reaction in general. Particularly under alkaline conditions, the phenolate ion is a better leaving group than an alkoxide in the transesterification of esters, because phenols are stronger acids than alcohols, and the acidity increases as the benzene nucleus carries electron-attracting substituents such as halogens or nitro groups [24]. The possibility that chlorophenol/fatty acid esters would remain unaffected during the transesterification procedures was therefore considered to be low. In a previous study [10], 90% of free chlorophenols dissolved in cyclohexane were recovered as acidic substances on liquid–liquid extraction with a pH 9 buffer. Hence esterified chlorophenols ought to be released from their fatty acid esters and the majority of them recovered in the acidic fraction.

As only a small part (< 1–15%) of the EOCl was recovered as acidic material after the transesterification procedures (Table II), it is therefore suggested that chlorinated phenolic compounds were of minor significance to the hydrolysable chlorinated compounds in the fish lipids studied. More direct methods should be chosen, however, if the amount of chlorine in esterified chlorophenolic compounds is to be assessed. A combination of the results given above with the general picture that the compounds representing EOCl have relatively high molecular weights (> 300 [2,8,9]) and are hydrolysable strongly supports the suggestion that the major portion of EOCl in fish is associated with chlorinated carboxylic acids bound in acylglycerols.

Detection of chlorinated FAMEs with ELCD

ELCD detected chlorinated but not unchlorinated FAMEs (Fig. 2). The detection limit (DL), defined as a peak height larger than twice the noise, found with GC–ELCD was about 50 pg of chlorine, which equalled 400 pg of monochloro- and 250 pg of dichlorostearic acid methyl esters (Fig. 2). GC–FID showed about the same DL (300 pg) for the reference substances as did ELCD. In spite of the high DL obtained with GC–ELCD (20–30 times higher than expected), possibly depending on an insufficiently pure make-up gas or excessive column bleeding, the results achieved are presented because of the novel application.

It was possible to detect halogenated compounds among FAMEs produced from the eel lipids A, containing 1200 ppm of EOCl (Fig. 3). The bromine concentration (EOBr) was only 0.2% of EOCl and all the detected compounds were, therefore, chlorinated substances. The ELCD response is almost proportional to the amount of chlorine injected and independent of the compound structure [25,26]. It was therefore possible to determine how much of the injected EOCl that was accounted for by the

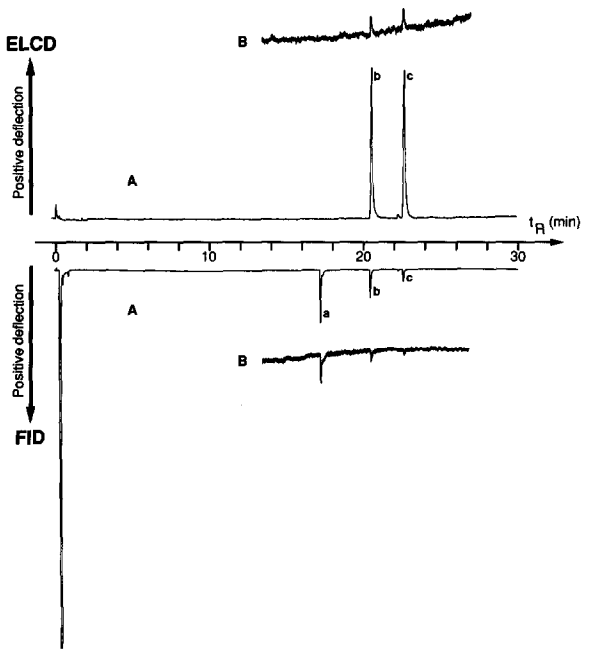

Fig. 2. Simultaneous detection with GC–ELCD and GC–FID of methyl esters of the reference compounds: (a) oleic acid, (b) monochlorostearic acid and (c) dichlorostearic acid. Chromatograms labelled A refer to an injection of 30 ng, 17 ng (1.8 ng chlorine) and 10 ng (1.9 ng chlorine) of compounds a, b and c, respectively. The inserted chromatograms labelled B, obtained at the limit of detection, refer to an injection of 0.74 ng, 0.42 ng (46 pg chlorine) and 0.25 ng (48 pg chlorine) of compounds a, b and c, respectively. t_R = Retention time.

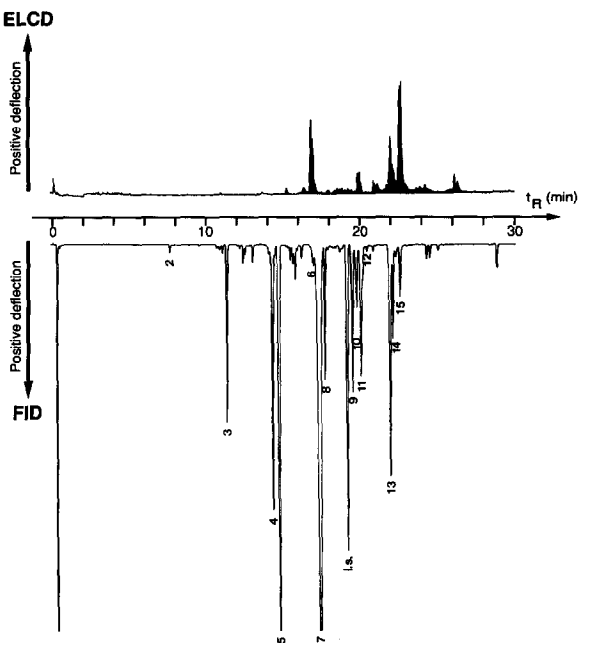

Fig. 3. Simultaneous detection with GC–ELCD and GC–FID of 7.2 μg of FAMEs from eel lipids containing 8.6 ng of organically bound chlorine. The dark area used for integration of the total ELCD response corresponds to 7.8 ng of chlorine. The FAMEs shown in the FID chromatogram, given by the numbers of carbon atoms and double bonds, are tentatively identified as 2 = 12:0, 3 = 14:0, 4 = 16:1, 5 = 16:0, 6 = 18:4, 7 = 18:2 and 18:1, 8 = 18:0, 9 = 20:5, 10 = 20:4, 11 = 20:1, 12 = 20:0, 13 = 22:6, 14 = 22:5, 15 = 22:1 and i.s. = 19:0 (internal standard).

GC–ELCD technique. An injection of 7.2 μg of FAMEs containing 1200 ppm of EOCl equalled 8.6 ng of chlorine. By comparison with the reference compounds, the total ELCD response of the eel sample corresponded to 7.8 ng of chlorine and the individual compounds contained less than 1 ng of chlorine. This means that ELCD accounted for 90% of the chlorine of the chlorinated substances in the eel lipids after their conversion into FAMEs. The ELCD chromatograms obtained with the other fish samples (30–60 ppm of EOCl) showed only minute peaks, which indicated that no solutes other than chlorinated compounds produced a measurable ELCD response. The other fish samples had 20–40 times lower EOCl concentrations than the eel caught in the receiving waters of the sulphite mill. If the chlorine in those lipids was distributed over a similar number of compounds, most of the constituents would be below the detection limit obtained.

The solution containing FAMEs (eels A) was treated three times with concentrated sulphuric acid to investigate whether the substances detected with ELCD were persistent compounds like PCBs. As this treatment eliminated the compounds that could be detected with ELCD (Fig. 4), the chlorinated substances were not regarded as persistent. It was also verified that the chlorinated reference substances (methyl esters) were not resistant to this treatment. Some small ELCD peaks were found with the sulphuric acid-treated eel sample after concentrating it by evaporation (Fig. 4). The pattern did not fit with that of a PCB mixture. The peaks were more likely derived from chlorinated fatty acids (see below).

The free fatty acids that were produced by enzymatic hydrolysis of the eel lipids had been sep-

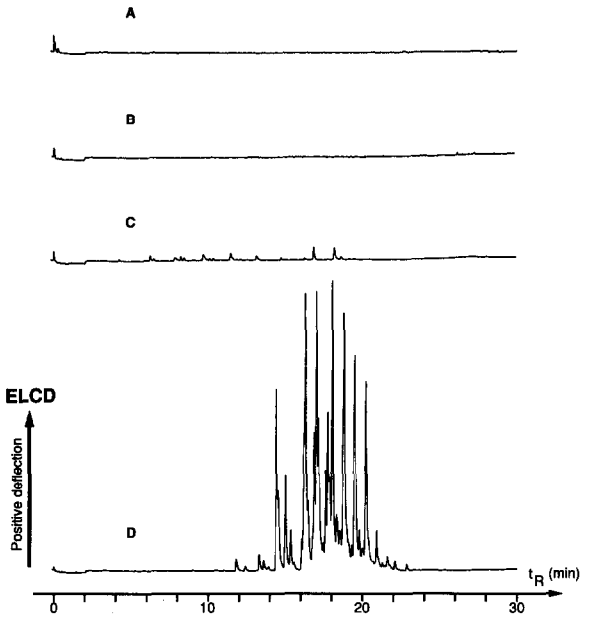

Fig. 4. GC–ELCD traces obtained after treating FAMEs of (A) the reference compounds (*cf.*, Fig. 2) and (B) the eel sample (*cf.*, Fig. 3) with concentrated H_2SO_4. After evaporation to a small volume, minor amounts of chlorinated material were found in the treated eel sample (C). Chromatogram D shows the ELCD response to 36 ng of PCBs (Clophen A50) equalling 18 ng of chlorine.

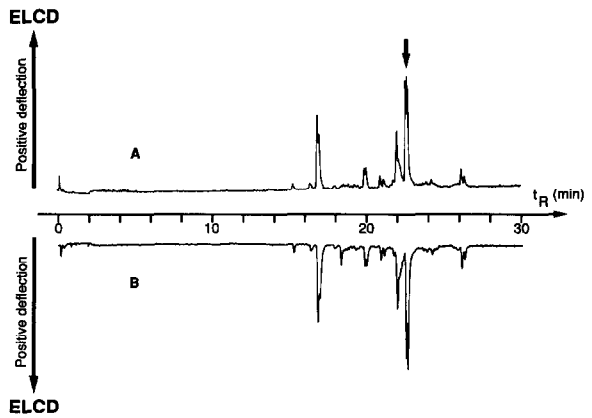

Fig. 5. (A) GC–ELCD of eel lipids subjected to alkaline transesterification and (B) GC–ELCD of FAMEs produced by acidic esterification of free fatty acids (separated from non-acidic compounds after enzymatic hydrolysis; *cf.*, Fig. 1). The arrow shows the retention time of methyl 9,10-dichlorostearate.

arated from non-acidic compounds by extraction into an alkaline buffer, followed by re-extraction into cyclohexane after acidification (Fig. 1). The free fatty acids were converted into FAMEs by acidic esterification and their GC–ELCD (Fig. 5) and GC–FID patterns were almost identical with those obtained after the alkaline transesterification. This implies that the chlorinated compounds detected with ELCD were chlorinated carboxylic acids. By co-injection, methyl 9,10-dichlorostearate was tentatively identified by its retention time as the largest peak in the ELCD chromatogram (Fig. 5). The presence of dichlorostearic acid in the eel lipids has recently been established by mass spectrometry [27]. Judging from the peak area, the concentration of dichlorostearic acid (having a chlorine content of 20%) in the eel lipid was calculated to be 600 ppm.

After the acidic esterification of the free fatty acids isolated from the hydrolysed eel lipids, one extra peak was found (Fig. 5), suggesting that this substance was degraded during the alkaline transesterification. Tinsley and Lowry [23] reported that 50% of a brominated stearic acid was degraded during alkaline hydrolysis whereas Sundin *et al.* [11] observed no degradation of dichlorostearic acid during alkaline transesterification from its triacylglycerol to the methyl ester. However, when working with yet unidentified compounds, the risk of producing artifacts must be considered. As HCl was used as a catalyst for this acidic esterification, it cannot be excluded that HCl had been added to some double bond in an unsaturated fatty acid. The acidic esterification with sulphuric acid might have been more appropriate.

The two isomers of the synthesized monochlorinated stearic acids, 9- and 10-monochlorostearic acid, could not be separated with the GC system used. Similar results were also reported by Haken and Korhonen [28], who studied the GC retention indices of different isomers of monochlorinated FAME. Using an SE-30 column, they found a negligible influence on the GC retention from the location of the chlorine when the chlorine was located at the mid-positions of a long-chain FAME. However, they found a considerable enhancement in the retention with chlorine in terminal (ω) or near-terminal positions.

Several compounds could be detected by ELCD after treating a solution of FAMEs (eels A) with

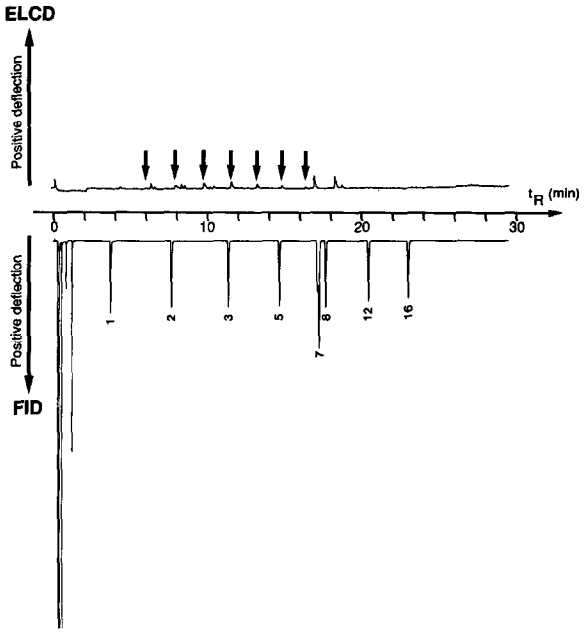

Fig. 6. GC–ELCD of FAMEs treated with H_2SO_4 (*cf.*, Fig. 4) and GC–FID of a synthetic mixture of FAMEs, containing members of a homologous series. The FID peaks correspond to 1 = 10:0, 2 = 12:0, 3 = 14:0, 5 = 16:0, 7 = 18:2 and 18:1, 8 = 18:0, 12 = 20:0 and 16 = 22:0. A possible homologous series is shown by arrows on the ELCD trace. The ELCD retention times seem to indicate that each compound is separated from the previous one by one CH_2 group.

concentrated sulphuric acid and thereafter concentrating the sample by evaporation. Their retention times were compared with the GC–FID pattern of a homologous series of $C_{10:0}$–$C_{22:0}$ FAMEs. The similar elution patterns of the homologous series and some of the ELCD-detectable compounds (Fig. 6) suggest that some of the latter compounds might belong to a homologous series of chlorinated fatty acids and that each compound is separated from the previous one by one CH_2 group. The compounds were not present after treating the reference compounds with concentrated acid and therefore did not originate from the sulphuric acid. It cannot be concluded if the compounds were degradation products from longer fatty acids, became detectable after destroying the major compounds with concentrated sulphuric acid or were reaction products formed from unsaturated fatty acids and inorganic HCl released from the eel lipids during the treatment with sulphuric acid.

By treating a sample with concentrated sulphuric acid, the persistent EOCl (EPOCl) can be assessed [2]. The proportion of EPOCl that can be identified by GC–ECD has been found to be in the range 40–100% for fish from a fiord area polluted by industrial effluents from magnesium production [29]. In tissues of seal caught off the Norwegian coast, the identified EPOCl has been found to be in the range 10–100% [30] and, in a recent study of salmon and eel lipids [6], the sum of chlorine in PCBs, DDT and DDE amounted to 7–36% of EPOCl. As ECD seems not to be a suitable detection method for chlorinated fatty acids (see below), it is possible that part of the unidentified EPOCl belongs to chlorinated fatty acids remaining after, or formed during, the treatment with concentrated sulphuric acid.

Detection of FAMEs with ECD

When working with GC–ECD, negative peaks are occasionally encountered in the chromatograms. An extreme result was obtained when FAMEs of eel lipids A were injected into the GC system and studied using ECD and FID in parallel. It was not possible to obtain any positive signal from ECD (Fig. 7) comparable to the ELCD response (Fig. 3). Owing to a high background with ECD, the unchlorinated FAMEs were less electron capturing and were therefore detected as negative peaks. This result was found both with the Varian detector and with a Carlo Erba Model 4160 gas chromatograph and a Model HT-25 electron-capture detector using argon–4% methane as the make-up gas and nitrogen as the carrier gas. The negative ECD response from unchlorinated FAMEs has been reported previously [31].

Sundin *et al.* [11] found a GC–ECD detection limit for methyl dichlorostearate of about 500 pg. In this study, the peak representing methyl dichlorostearate changed from a positive to a negative appearance on reducing the injected amount to 2.5 ng (Fig. 7). This problem was encountered after injecting several samples containing fish FAMEs. It was not investigated whether the problem resulted from residues of the transesterification reagent or from other solutes. However, the problem must be solved otherwise ECD is not suitable for the detection of chlorinated fatty acids in fish lipids.

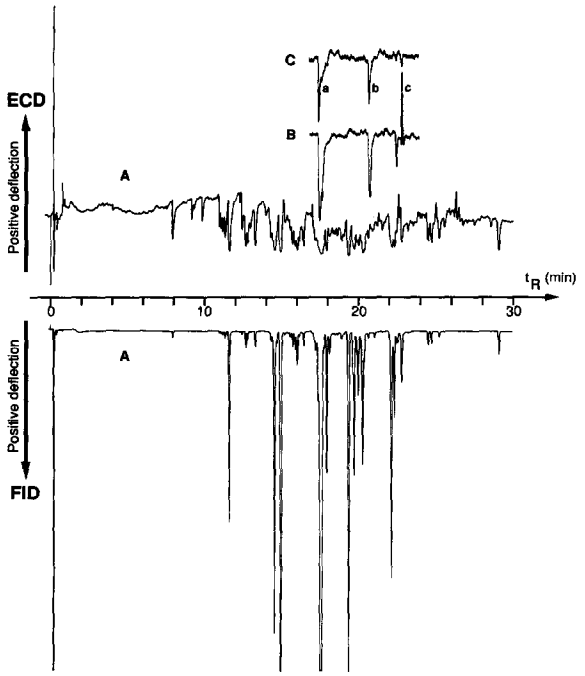

Fig. 7. Simultaneous detection with GC–ECD and GC–FID. (A) 4.5 μg of FAMEs of eel lipids (*cf.*, Fig. 3). The inserted ECD traces B and C were obtained with methyl esters of the reference compounds (a) oleic acid, (b) monochlorostearic acid and (c) dichlorostearic acid [(B) 30, 17 and 10 ng; (C) 7, 4 and 2.5 ng].

General considerations

This work indicates that chlorinated carboxylic acids bound in acylglycerols make up an important part of EOCl in fish tissues and that a fungal lipase can hydrolyse such substances. The chlorinated carboxylic acids are presumably of fatty acid character, as they have similar GC retention times to the normal fish FAMEs. This is the first report in which as much as 90% of EOCl in fish lipids could be detected after GC separation and, thus, the GC–ELCD technique can be successful in detecting new halogenated substance groups in environmental samples.

In the first study on the hydrolysable properties of organochlorine compounds in fish and sediments [10], it was assumed that short-chain fatty acids containing chlorine were liberated by the hydrolysis and then lost in the washing procedure, thereby causing a considerable loss of EOCl after hydrolysis. In this study, the ELCD chromatograms revealed that there was only a small difference between FAMEs produced by alkaline transesterification of the untreated eel lipids and FAMEs produced from free fatty acids that were isolated after an enzymatic hydrolysis (Fig. 5). As the ELCD chromatograms of FAMEs of the eel lipids A could be estimated to represent about 90% of the EOCl, the loss of EOCl after the enzymatic hydrolysis, *ca.* 40% (Table I), more likely depended on adsorption on the immobilized enzyme or to its carrier.

The incorporation of chlorinated fatty acids in fish lipids has been overlooked, although some workers have discussed their possible occurrence [10,32,33]. However, in connection with investigations on chlorinated cake flour, it has been demonstrated that chlorinated fatty acids can be taken up and distributed in rat tissues and that the substances can be transferred from mother to suckling rats and also, to some extent, to the foetuses via the placenta (reviewed by Cunningham [34]).

The chlorinated carboxylic acids found in the eel lipids were probably derived from compounds in the sulphite mill bleach effluents. Fatty acids in the wood are dissolved during the pulping processes and chlorinated fatty acids can occur in bleach liquors if the pulp is not thoroughly washed before bleaching. Dichlorostearic acid has been detected in effluents from Canadian kraft mills [35,36] and di- and tetrachlorostearic acids have been found in sediments from the receiving waters of three Scandinavian kraft mills [7]. A modern kraft mill normally has an oxygen prebleaching stage ahead of the chlorine and chlorine dioxide stages. The oxygen stage effectively reduces the concentration of fatty acids and resin acids in the pulp [37], thereby reducing the formation of chlorinated acids in the effluents. However, it ought to be considered whether the chlorinated fatty acids in fish are directly taken up from the effluents or are metabolites from other compounds formed during the bleaching processes. The Coulson electrolytic conductivity detector has been used for the GC detection of brominated fatty acids in rat tissues after administering di- and tetrabromostearic acids to rats via the food [38]. In addition to the administered substances, di- and tetrabrominated metabolites (palmitic and myristic acids) were found. At least part of the chlorinated carboxylic acids indicated by GC–ELCD in this work might be metabolites produced in the fish or in its prey organisms.

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