



Chlorinated Fatty Acids in Freshwater Fish and Some Biological Effects of Dichlorostearic Acid

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Abstract

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Chlorinated fatty acids (CFAs), major constituents of extractable, organically bound chlorine in biota, are found in almost all fish studied in this respect. The concentration of CFAs in fish varies considerably from some micrograms per gram of lipids in fish from remote areas up to more than two thousand micrograms per gram of lipids in fish from the vicinity of chlorine bleached kraft mills.

CFAs were liberated from fish lipids as the corresponding methyl esters and subjected to enrichment. The methylated CFAs were studied by gas chromatography with electrolytic conductivity detection and/or mass spectrometry.

A considerable diversity of CFAs were found in fish, e.g. more than twenty were indicated in pike from Latvian lakes. However, only part of the CFAs could be identified. Pattern of CFAs differ considerably between fish from different areas. Thus, the patterns of CFAs in previously studied eel caught in Idefjord differ from the CFA patterns of pike from Latvian lakes and also from that of perch from Latvian rivers. The main CFAs in pike are likely chlorohydroxy fatty acids and in perch chlorinated, possibly sulphur-containing carboxylic acids. In the study of pike, CFAs were released from all lipid classes considered: steryl esters, triacylglycerols, and phospholipids.

Because an enrichment is usually needed to make CFAs in lipids detectable, their quantification may be difficult. A method to facilitate quantitative studies was developed, where the enrichment factor is calculated using cholesterol.

Dichlorostearic acid was taken up by rats via food and were distributed within different organs with the highest concentration in heart lipids and the lowest in muscle lipids. Dichlorostearic acid was metabolised in rats yielding dichloropalmitic and dichloromyristic acids. Some shorter-chain metabolites, possibly including dichlorotridecanoic acid were found in the liver lipids. CFAs in the lipid extracts of rat tissues were studied also by liquid chromatography with electrospray ionisation mass spectrometry.

In a test for mutagenicity, the Ames test, dichlorostearic acid did not show any adverse effects. In contrast, dichlorostearic acid caused inhibition of mutagenicity of some indirectly-acting mutagens, possibly by the inactivation of Cytochrome P450 enzymes. Certain effects on the ability of rat enzymes to activate indirectly acting mutagens by the same acid was found also in *in vivo* studies. A possible explanation to this observation might be an interaction of dichlorostearic acid with membrane bound enzymes or changes in the membrane lipid composition resulting from the exposure to dichlorostearic acid.

Key words: cell membranes, chlorinated fatty acids, Cytochrome P450 enzymes, electrolytic conductivity detection, gas chromatography, liquid chromatography, mass spectrometry, mutagenicity, perch, pike, rats

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Preface

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. Vereskuns, G., Sundin, P., Wesén, C., Mu, H., Björn, H., Klavins, M., Göransson, A. & Odham, G. 1999. Chlorinated fatty acids and persistent organic pollutants in pike (*Esox lucius*) from Latvian lakes and from the Baltic. Submitted.
- II. Vereskuns, G., Sundin, P., Nilsson, E., Olsson, A., Valters, K. & Wesén, C. 1999. Chlorinated fatty acids and related compounds in perch (*Perca fluviatilis*) from Latvian rivers. Manuscript.
- III. Vereskuns, G., Wesén, C. & Sundin, P. 1999. Using cholesterol to calculate the enrichment factor of chlorinated fatty acids after pre-concentration. Submitted.
- IV. Vereskuns, G., Wesén, C., Skog, K. & Jägerstad, M. 1998. Inhibitory effect of *threo*-9,10-dichlorostearic acid on the mutagenic activity of MeIQx, 2-AF and B[a]P in the Ames/*Salmonella* test. *Mutation Research* 416: 149-157.
- V. Vereskuns, G., Wesén, C., Skog, K. & Sundin, P. 1999. Effects of dichlorostearic acid on organs and enzyme activity in rats. Manuscript.

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Abbreviations

AES	atomic emission spectrometry
CFAs	chlorinated fatty acids
CI	chemical ionisation
DDE	dichloro-2,2-bis(4-chlorophenyl)ethene
DDT	trichloro-2,2-bis(4-chlorophenyl)ethane
ECD	electron capture detection
EI	electron ionisation
ELCD	electrolytic conductivity detection
EOBr	extractable, organically bound bromine
EOCl	extractable, organically bound chlorine
EOI	extractable, organically bound iodine
EOX	extractable, organically bound halogens
ESI	electrospray ionisation
FAB	fast atom bombardment
FAMES	fatty acid methyl esters
FID	flame ionisation detection
GC	gas chromatography
HPLC	high performance liquid chromatography
IR	infrared spectroscopy
MS	mass spectrometry
NAA	neutron activation analysis
NICI	negative ion chemical ionisation
NMR	nuclear magnetic resonance spectroscopy
PCBs	polychlorinated biphenyls
PICI	positive ion chemical ionisation
POPs	persistent organic pollutants
SIM	selective ion monitoring
TLC	thin layer chromatography
UV	ultra violet
XSD	halogen selective detection

Introduction

For some decades chlorinated compounds found in the environment have attracted considerable attention. One reason is the environmental harm caused by persistent organochlorine pollutants such as DDT and its metabolites, PCBs, chlorinated phenols, and chlorinated dibenzofurans and dioxins. However, these compounds constitute only a small part of the extractable, organically bound chlorine (EOCI), which has been widely used as an expression of the chlorine pollution level (Lunde & Steinnes, 1975; Carlberg et al., 1987; Newsome et al., 1993). Only in the early nineties it was found that up to 95% of the EOCl in biota can be accounted for by chlorinated fatty acids (CFAs) (Wesén, 1995; Mu, 1996; Milley et al., 1997). Also brominated (Lunde, 1972; Tinsley & Lowry, 1980; Lam et al., 1989; Carballeira & Emiliano, 1993; Garson et al., 1993, 1994; Carballeira & Reyes, 1995) and iodinated (Kawano et al., 1995) lipids have been found in the environment but in concentrations much lower than that of chlorinated lipids (Gether et al., 1979; Kannan et al., 1999).

In most environmental samples the EOCl concentration ranges from 20 to 60 $\mu\text{g/g}$ lipids (Lunde et al., 1976; Gether et al., 1979; Martinsen et al., 1988; Håkansson et al., 1991; Wesén, 1995; Mu, 1996) with some exceptions such as the pathogenic fungus *Verticillium dahliae* (1200 $\mu\text{g/g}$ lipids; Stepanichenko et al., 1977) and fish from the areas polluted by pulp mill effluents (up to 2000 $\mu\text{g/g}$ lipids; Carlberg et al., 1987; Håkansson et al., 1991; Wesén et al., 1992a) or near the discharge outfall of a former alkali-chlorine facility at a coastal area in USA (up to 2170 $\mu\text{g/g}$ lipids; Kannan et al., 1999). The highest concentration of EOCl in biota reported (3038 $\mu\text{g/g}$ lipids; Kannan et al., 1999) was found in red-winged blackbirds near that site.

Gas chromatography (GC) with halogen or chlorine selective detection has most commonly been used to detect and identify CFAs in different samples. In recent decades electrolytic conductivity detection (ELCD) has been found to be very useful to determine halogenated fatty acids (Conacher et al., 1984; Lawrence et al., 1984; Wesén, 1995; Heikes, 1993; Wesén, 1995; Mu, 1996). Also GC with mass spectrometry (MS) has been widely used to identify CFAs (Gibson et al., 1986; McKague & Reeve, 1991; Sundin et al., 1992; Heikes, 1992; Wesén et al., 1995; Mu, 1996; Milley et al., 1997). Other detectors that have been used in studies of CFAs are the electron capture detector (Komo-Suwelack et al., 1983), the atomic emission spectrometer (Pedersen-Bjergaard & Greibrokk, 1993; Jonsson et al., 1995), infrared spectrometer (White & Hager, 1977; McKague & Reeve, 1991) and nuclear magnetic resonance spectrometer (Stepanichenko et al., 1977; McKague & Reeve, 1991). To evaluate the total concentration of extractable, organically bound halogens (EOX), and EOCl in particular, without identification of individual compounds neutron activation analysis (NAA) is the most commonly used technique (Lunde & Steinnes, 1975; Gether et al., 1979;

Martinsen et al., 1988; Håkansson et al., 1991; Wesén, 1995). In recent years the probe injection dual-microplasma atomic emission spectrometry (AES) has been introduced as an alternative to NAA (Asp et al., 1997).

The major single source of CFAs is probably chlorine bleached pulp and paper production (Leach & Thakore, 1977; Easty et al., 1978; Voss & Rapsomatiotis, 1985; Neilson et al., 1991; Wesén, 1995) and, possibly, alkali-chlorine processes (Kannan et al., 1999). CFAs may be synthesised also during some other humans activities such as water, meat, poultry and fish disinfection with chlorine containing agents (Cunningham & Lawrence, 1979; Cunningham, 1980; Fukayama et al., 1986; Gibson et al., 1986) or bleaching of flour in order to improve the baking quality (Komo-Suwelack et al., 1983; Wei et al., 1984; Fukayama et al., 1986; Heikes, 1992, 1993). A seminatural way of formation may be the synthesis of CFAs by certain organisms from man-made chlorinated compounds such as chloroalkanes (Madeley & Birtley, 1980, Murphy & Perry, 1987; Omori et al., 1987). Furthermore, it has been postulated that CFAs also may be synthesised naturally (Stepanichenko et al., 1977; White & Hager, 1977; Neidleman & Geigert, 1986).

A number of studies have been done on the toxicology of CFAs. It seems that CFAs affect reproduction related processes (Cherr et al., 1987; Håkansson et al., 1991; Björn et al., 1998a) and have other negative effects, such as a reduction of the weight gain and an increase in weight of some organs in rats (Cunningham & Lawrence, 1977b, 1978). Although CFAs have negative effects on certain organisms these acids do not seem to activate the detoxifying enzyme systems (Håkansson et al., 1991; Goksøyr & Larsen, 1993), that are commonly studied with respect to xenobiotics such as PCBs. Moreover, CFAs can be taken up by food, assimilated and transferred into organisms similarly to non-chlorinated fatty acids (Cunningham & Lawrence, 1977abc; Conacher et al., 1984; Ewald et al., 1996; Mu, 1996; Björn, 1999). The adverse effects of CFAs in combination with their wide distribution in the environment make them interesting and important objects of study.

Objectives

The aims of this study were to characterise unknown chlorinated compounds of fatty acid character in fish lipids as well as to study possible harmful effects of CFAs to living organisms. The detection and identification of chlorinated compounds in fish lipids were focused on fish from Latvia, employing gas chromatography with electrolytic conductivity, mass spectrometric, or electron capture detection [Paper I & II]. To facilitate the quantification of CFAs in fish samples after selective enrichment (Mu et al., 1996b), a method using cholesterol present in fish lipids was developed [Paper III]. Furthermore the mutagenic and

antimutagenic properties *in vitro* [Paper IV], and metabolism and some toxic effects *in vivo* [Paper V] of CFAs were studied.

Analysis of chlorinated fatty acids

CFAs in environmental samples are hard to identify and even to detect. Mainly it is because the concentrations of CFAs are very low in comparison to ordinary, non-chlorinated fatty acids. Furthermore, they are bound in lipids, which make impossible straight-forward clean-up methods such as removal of the lipid matrix by sulphuric acid treatment in the analysis of PCBs and similar compounds (Jensen et al., 1983). Consequently, even if the detector selectivity to CFAs is high, the retention indices of chlorinated compounds in a sample may be affected by co-eluting compounds. In case an MS is used to study CFAs the co-eluting, far more abundant non-chlorinated fatty acids may obscure spectra. In order to avoid this, a fractionation and/or an enrichment procedure (Stepanichenko et al., 1977; Wesén et al., 1995b; Mu et al., 1996b; Milley et al., 1997) may be employed to increase the concentrations of CFAs in the extracts.

Enrichment of CFAs

Transmethylation of the lipids extracted from the fungus *Verticillium dahliae* followed by fractionation by using complexation with urea, crystallisation from acetone at -60 °C, thin layer chromatography (TLC) on silica gel containing AgNO₃, column chromatography on Al₂O₃ and preparative TLC on Al₂O₃ was used to prepare a sample for ¹³C-NMR, IR spectroscopy and MS studies, which resulted in the identification of 9,10-dichlorostearic acid (Stepanichenko et al., 1977). The method used by Stepanichenko and co-workers has been recognised by English-speaking readers only in the form as an abbreviated version in Chemical Abstracts regarding the brominated analogues (Gusakova & Umarov, 1977) without any detail of the complexation procedure. Therefore, the method developed by Mu and co-workers (Mu et al., 1996b) actually turned out to use a very similar concept, but has the advantage of being less complex and time consuming, which otherwise is a draw-back in routine analyses when large number of samples are to be processed. In brief, the presently used method for enrichment of CFA methyl esters (Mu et al., 1996b) is based on a consecutive treatment with silver nitrate and urea, leading to an enrichment of CFAs by up to a factor of 30. However, CFAs must be saturated and bulky enough not to form complexes with silver ions and urea, respectively. Methyl esters of monochlorinated fatty acids have been found to form the urea inclusion complexes (Mu et al., 1996b) and are therefore removed, at least partly, from the samples during the enrichment. Furthermore, a risk remains that some additional

species of chlorinated fatty acids may be removed from a sample during the enrichment because of the presence of double bonds or of insufficient bulkiness.

Sometimes a purification of a sample by solid phase extraction using silica is recommended before the enrichment, especially when the sample contains oxidised materials that can negatively affect the efficiency of the urea treatment (Wesén et al., 1995a; Paper II, III). Such a purification may not only improve the urea complexation step but also increases the total efficiency of the enrichment by removing undesirable compounds such as oxidised lipids and cholesterol from a sample (Paper II).

Some extra purification steps can be necessary also after the enrichment of CFAs. Thus, Mu and co-workers (Mu et al., 1996b) used silica gel TLC before the GC/MS and GC/ELCD studies to separate chlorinated FAMES from monounsaturated non-chlorinated ones still remaining after the enrichment. Milley and co-workers (Milley et al., 1997) used gel permeation chromatography to purify chlorinated FAMES after their enrichment by urea complexation. Also preparative GC, as described in Wesén et al., (1995b), should be feasible to use to fortify the enrichment of CFAs.

However, there are also some disadvantages in using an enrichment. After the enrichment it is hard to perform quantitative analysis of CFAs due to difficulties to determine an exact extent of enrichment. Therefore, a procedure for enrichment efficiency calculation, such as that described in Paper III, may be advantageous for the quantification of CFAs in environmental samples.

Detection of CFAs

There are several compound-specific determination procedures used for CFAs. These procedures usually employ gas chromatographic separation of chlorinated FAMES or other suitable derivatives with electron capture detection (ECD), electrolytic conductivity detection (ELCD), halogen selective detection (XSD), AES, or MS. Some other techniques such as plasma spray MS (Sundin et al., 1992) or electrospray MS (Paper V), following liquid chromatographic separation, may also be used to detect CFAs.

The electron capture detector is commonly used in the analysis of persistent organochlorine pollutants after their separation by GC, but has also been used to study chlorinated FAMES in bleached flour (Komo-Suwelack et al., 1983). However, due to negative peaks produced by compounds lacking electrophilic functional groups, as is the case with non-chlorinated FAMES, it is problematic to use this detector for CFAs in environmental samples (Wesén et al., 1992). It has also been found that the ECD detection limit for dichlorostearic acid is relatively

high (about 0.5 ng) with no response for the monochlorinated analogue (Sundin et al., 1992).

The electrolytic conductivity detector is an element selective detector (Piringer & Pascalau, 1962; Piringer et al., 1964; Coulson, 1965,1966; Anderson & Hall, 1980), therefore, in comparison with the ECD, the ELCD is more suitable for the detection of CFAs in complex matrices such as environmental samples. It has a dynamic linear range of more than five orders of magnitude and its response is little affected by the molecular structure of compounds (Piringer & Wolf, 1984; Mu et al., 1999). GC/ELCD has been used in studies of the metabolism of CFAs in rats (Conacher et al., 1984; Lawrence et al., 1984; Paper V) and also to detect chlorinated fatty acids in lipids of fish (Wesén, 1995; Mu, 1996; Björn, 1999; Paper I, II), porpoise (Björn, 1999), and bleached flour (Heikes, 1993). The ELCD detection limit for chlorinated FAMES is about 50 pg of chlorine, which corresponds to about 250 pg of methyl dichlorostearate or 400 pg of methyl monochlorostearate (Wesén et al., 1992). However, the ELCD is not a chlorine selective detector, but a halogen selective device giving similar response also to bromine and iodine (Coulson, 1965). On the other hand, it has been observed that in the lipids of aquatic organisms extractable, organically bound bromine (EOBr) usually corresponds to less than 0.5% of the EOCI (Gether et al., 1979; Wesén et al., 1992b) and that the extractable, organically bound iodine (EOI) in biota usually makes up a lower proportion than EOBr (Gether et al., 1979, Kawano et al., 1995; Kannan et al., 1999; Kawano et al., 1999). Therefore, ELCD detectable compounds extracted from biota most likely represent chlorinated compounds. Consequently, during studies of CFAs in lipids it should be possible to consider the ELCD mainly as a chlorine selective detector.

Also the halogen selective detector (XSD) can be used to detect CFAs as their methyl esters after GC separation. This detector has been used to detect CFAs and their metabolites in human cell lipids (Gustafson-Swärd et al., 1999). During studies of model compounds of chlorinated FAMES it showed similar sensitivity and selectivity as the ELCD (Åkesson-Nilsson et al., 1999).

In contrast to the ECD, the ELCD and the XSD, the atomic emission spectrometer (AES) is a truly element selective detector. The GC/AES detection limit for bromine and chlorine can be about 1 pg/s (Pedersen-Bjergaard & Greibrokk, 1993) with a linearity of more than 3 orders of magnitude. GC/AES has been used to confirm the presence of chlorine and bromine in halogenated aliphatic acids produced by chlorination of humic acids in the presence of bromide ions (Peters et al., 1994) and to detect organochlorine and organobromine compounds in drinking water (Pedersen-Bjergaard & Greibrokk, 1993).

GC/MS is widely used in fatty acid analysis. Different ionisation techniques, such as electron ionisation (EI) (e.g. Mu et al., 1996a), positive and negative ion chemical ionisation (PICI and NICI) (e.g. Sundin et al., 1992), and fast atom

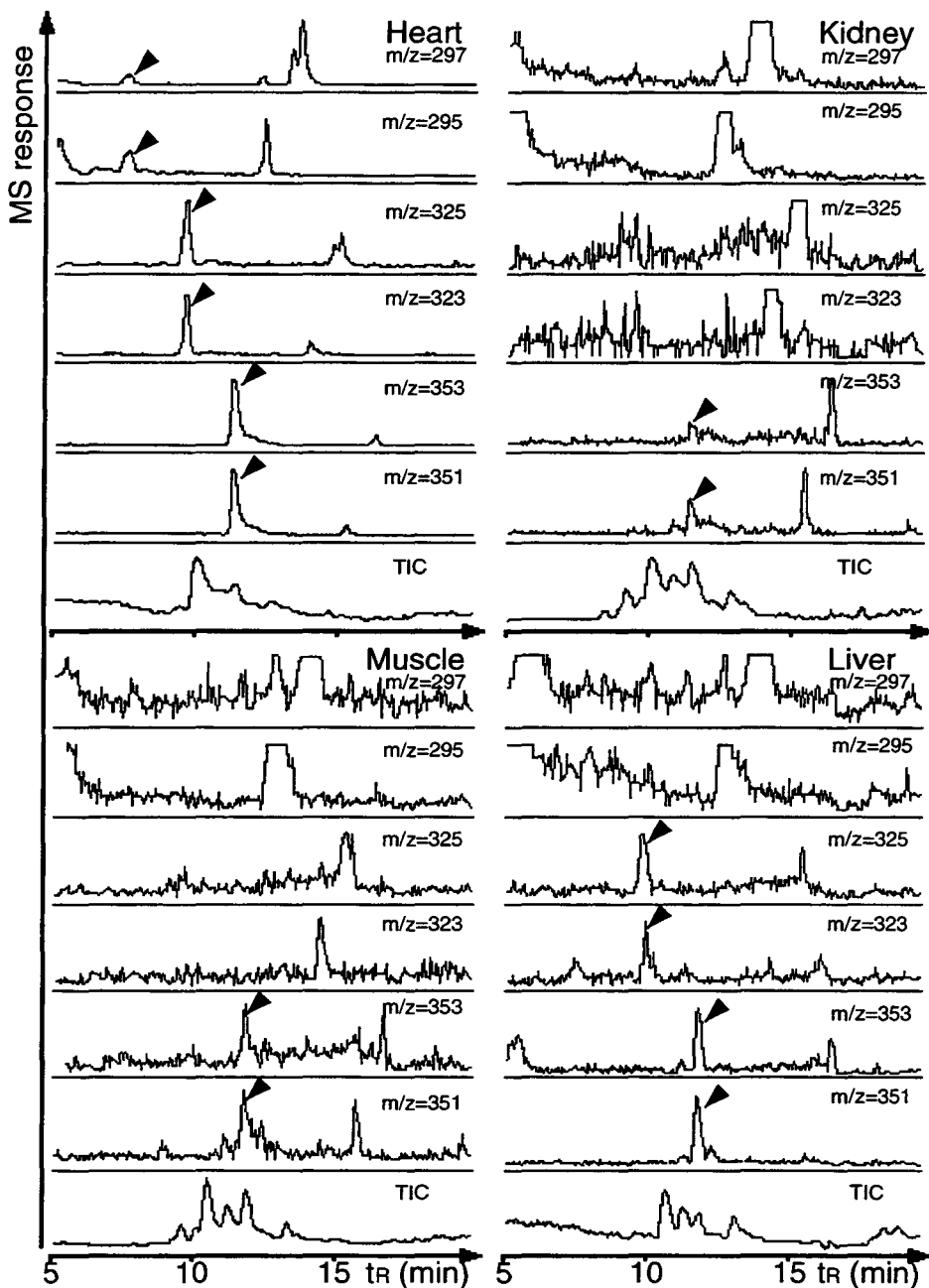


Fig. 1. HPLC/MS extracted ion fragmentograms and total ion chromatograms of lipids from different organs of a rat exposed to dichlorostearic acid. Presence of dichlorostearic ($m/z=351$ and 353), dichloropalmitic ($m/z=323$ and 325), and dichloromyristic ($m/z=295$ and 297) acid was studied by using the corresponding $[M-1]^-$ ions.

bombardment (FAB) (e.g. Gibson et al., 1986), have been used in studies of halogenated fatty acids. However, due to the large excess of non-chlorinated FAMES in the samples studied the MS analyses, especially EIMS, of chlorinated FAMES in the full scan mode can be disturbed by non-chlorinated FAMES (Wesén et al., 1995b; Mu, 1996; Paper I). Therefore, selective ion monitoring (SIM) is preferably used to detect CFAs in environmental samples, by monitoring characteristic ions. This is particularly useful when CI is used, which is a soft ionisation technique often giving rise to molecular weight related ions of CFAs (Heikes, 1992; Sundin et al., 1992; Wesén et al., 1995b; Mu et al., 1996ab; Mu, 1996; Milley et al., 1997). However, only those CFAs will be detected for which ions to monitor are known, while unknown CFAs remain undetected. On the other hand, all chlorinated compounds including CFAs may be detected if ions with $m/z=35$ and 37 , which correspond to chlorine isotopes, are monitored (Milley et al., 1997).

Although GC with different suitable detectors is the most commonly used technique to detect CFAs also HPLC/MS (Sundin et al., 1992; Paper V) or even better HPLC/MS-MS may be used for the same purpose. Such techniques allow for the detection of CFAs incorporated in glycerol- or phospholipids. However, a certain CFA may be bound in several different lipid species, each under the detection limit, and only the free CFAs may be possible to detect (Figure 1; Paper V).

Identification of CFAs

CFAs may be identified after GC separation of the proper derivatives and by comparing with the results from separation of the appropriate reference compounds, or by using retention indices (Komo-Suwelack et al., 1983; Conacher et al., 1984; Heikes, 1993; Mu et al., 1996ab; Milley et al., 1997; Björn, 1999; Gustafson-Svärd et al., 1999; Paper I, II, V). For such identification any of the detectors suitable for CFAs may be used, however, chlorine or at least halogen selective detectors are preferable to exclude interference of non-chlorinated fatty acids (Wesén et al., 1992).

The identification from the corresponding mass spectra obtained by one or several ionisation or scanning techniques is the most common method used today in studies of CFAs. In the EI mode, the MS is normally operated at an ionisation potential of 70 eV which provides sufficient energy for causing the characteristic fragmentation. EI mass spectra have been used to identify methyl esters after their separation by GC (Remberger et al., 1990; Sundin et al., 1992; Wesén et al., 1995b; Mu et al., 1996ab). However, using EIMS, the excessive fragmentation of chlorinated fatty acids with production of no or very few chlorine containing fragments is a major disadvantage in the search for trace amounts of CFAs in environmental samples. Yet, EIMS can be used in identification of CFAs in

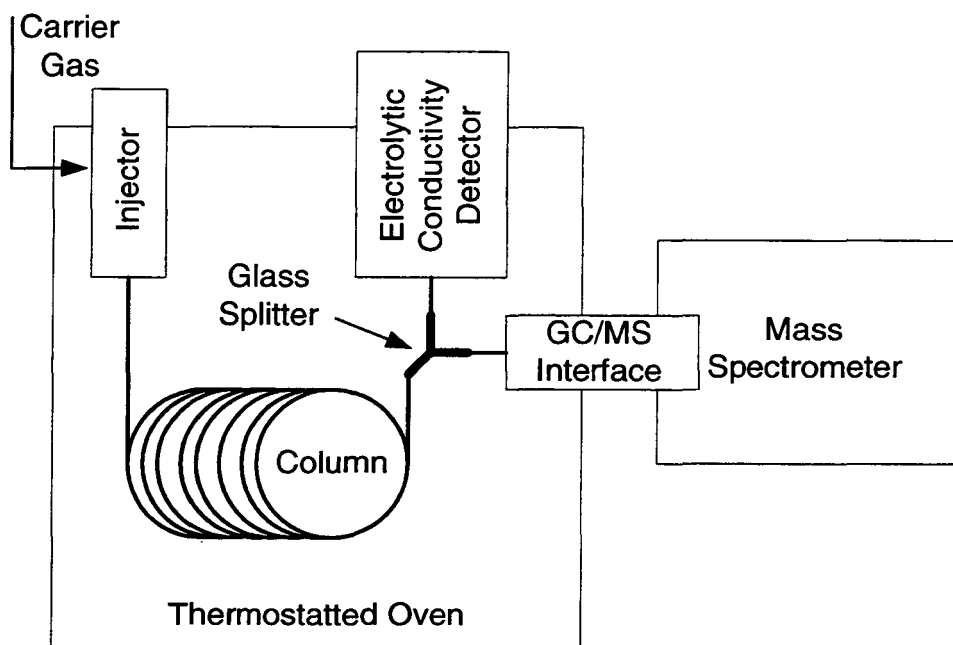


Fig. 2. Schematic drawing of the gas chromatograph with electrolytic conductivity detector and mass spectrometer.

combination with other techniques (Jonsson et al., 1995) or after extensive clean-up of a sample (Stepanichenko et al., 1977).

Much better results regarding the identification of CFAs have been obtained by using CIMS, which produce evidence of molecular ions (Heikes, 1992; Sundin et al., 1992; Wesén et al., 1995a; Mu et al., 1996ab; Mu, 1996; Milley et al., 1997; Paper I, II). Both PICI and NICI with different reagent gases have been used to identify CFAs in various samples. In order to support the identification of CFAs, high resolution SIM PICIMS has also been used (Mu et al., 1996a). However, little information about the structure of CFAs can be obtained by using CIMS due to the soft ionisation, which produces no or little fragmentation. Therefore, combinations of different ionisation methods and conditions are most advantageous for the identification of CFAs (Mu, 1996; Milley et al., 1997).

MS with electrospray ionisation (ESI), which also is a soft ionisation method, is commonly used in combination with HPLC. CFAs as free fatty acids or bound in complex lipids such as acylglycerols or phospholipids may be identified in HPLC/ESIMS studies by monitoring the corresponding ions, e.g. $[M-1]^-$ when using negative mode ESI (Paper V).

Other techniques also have been employed to identify CFAs in environmental samples. Infrared spectroscopy (IR) was used in the identification of CFAs in lipids of jellyfish (White and Hager, 1977) and a fungus (Stepanichenko et al., 1977). H-NMR was used to verify the identity of diastereomers of methyl 9,10-dichlorostearate utilised as reference (Wesén et al., 1995b) and ¹³C-NMR was employed in the identification of CFAs in lipids extracted from a fungus (Stepanichenko et al., 1977). IR, NMR and CIMS have been used to identify products of aqueous chlorination of linoleic acid (McKague & Reeve, 1991).

Retention indices (Peng, 1994; Mu et al., 1996ab) and column difference values (Mu et al., 1996a) may provide structural information of CFAs. GC studies of different derivatives of CFAs (Mu et al., 1996b; Paper II) or the operation of two detectors in parallel (Figure 2; Mu et al., 1996a; Paper II) can produce additional information facilitating the identification of CFAs.

Production of chlorinated fatty acids and occurrence in fish

Fatty acids including unsaturated ones, such as oleic and linoleic acids are present in wood extracts (Holmbom & Ekman, 1978; McKague & Reeve, 1991) and have been found in pulp mill discharges (Leach & Thakore, 1977; Voss & Rapsomatiotis, 1987). Chlorination of these unsaturated acids in pulp bleaching processes can lead to the formation of isomers of di- and tetrachlorinated stearic acids, and dichlorinated oleic acids (Leach & Thakore, 1977; McKague & Reeve, 1991). These acids as well as their shorter chain analogues have been found in eel caught near the pulp mill at the Idefjord (Mu et al., 1996a). The relation between CFAs in fish and pulp mill effluents has been discussed previously (Wesén, 1995; Mu et al., 1996ab). It has been suggested that CFAs with fourteen and sixteen carbon atoms in the chain may be produced by β -oxidation of CFAs with eighteen carbon atoms in the chain (Mu, 1996). The presence of dichlorotridecanoic acid in eel lipids might be explained by microbial degradation of CFAs of even carbon numbers (Björn et al., 1998b) or by chemical degradation and chlorination of fatty acids during pulp production.

There are also indications that CFAs may be synthesised in organisms (White & Hager, 1977; Stepanichenko et al., 1977). CFAs may be formed by the activity of chloroperoxidases that use chloride ions to produce organohalogenes (Neidleman & Geigert, 1986; Carr et al., 1996). It has also been found that rainbow trout can degrade chlorinated paraffins into smaller chlorinated molecules that may be incorporated into fatty acids through other biochemical pathways (Madley & Birtley, 1980). Chloroparaffins can also undergo co-metabolic degradation by *n*-alkane-degrading bacteria, leading to the production of a variety of chlorinated

carboxylic acids (Omori et al., 1987). Certain hydrocarbon-utilising microorganisms can oxidise 1-chloroalkanes, producing ω -CFAs which can be incorporated in cellular lipids (Murphy & Perry, 1987; Curragh et al., 1994; Hamilton et al., 1995). Jernelöv (1989) suggested that CFAs might be produced in the natural fatty acid synthesis if low-molecular weight organochlorine precursors are present.

CFAs have been found in both freshwater and marine fish from Alaskan, Baltic, and Scandinavian waters (Wesén et al., 1992b, 1995b; Mu, 1996; Mu et al., 1996a; Björn, 1999; Paper I, II). The concentration of CFAs has been found to be higher in phospholipids than in acylglycerols (Björn et al., 1998b). Particularly, CFAs in fishes from the vicinity of chlorine bleached pulp mills may have anthropogenic origin or may be metabolites from anthropogenic precursors. On the other hand, CFAs found in fish from remote areas may be produced by natural pathways, e.g. by metabolic activities involving haloperoxidases (Neidleman & Geigert, 1986; Mu et al., 1997). Dichlorostearic acid can be formed also by the action of ultraviolet (UV) light on a mixture of oleic acid with DDT or methoxychlor (Schwack, 1988). It is possible that CFAs may be formed by the presence of these chemicals on leaf or water surfaces where both lipid moieties and UV light are at hand. It is also possible that CFAs may be taken up by fish from sediments where these acids could be formed from chloroalkanes under activities of hydrocarbon-utilising microorganisms (Murphy & Perry, 1983, 1987; Curragh et al., 1994; Hamilton et al., 1995).

More than twenty CFAs were found in pike from Latvian lakes (Paper I). However, the FAMES released from the pike lipids were found to differ from those found in the eel from Idefjord (Paper I). GC/MS studies of CFAs from the pike indicated the presence of chlorohydroxy fatty acids (Paper I). It has been discussed whether chlorohydroxy fatty acids may be formed during the reaction of hypochlorous acid with unsaturated fatty acids (Cunningham & Lawrence, 1979). Such interactions may happen during the aqueous bleaching of pulp and paper when hypochlorous acid is formed (pH dependent) that may react with unsaturated fatty acids present in wood.

GC/ELCD studies of chlorinated FAMES released from lipids of perch from Latvian rivers (Paper II) and lakes (unpublished) showed that CFAs in the perch differed from those found in the eel and pike. After GC/MS studies of the chlorinated FAMES it was suggested that at least part of the CFAs in the perch lipids are chlorinated, possibly sulphur-containing carboxylic acids (Paper II). Similar compounds have been found in lipids of porpoise from the Swedish west coast (Wesén, personal communication). Different chlorosulpholipids have been found in the phytoflagellate *Ochromonas danica* (Mooney et al., 1972; Mooney & Haines, 1973, Elovson, 1974). Patent right has also been claimed for the manufacture of chlorosulpholipids to be used in the leather and fur industry (Mu et al., 1997).

Uptake and metabolism of chlorinated fatty acids in living organisms

Uptake of CFAs

It is not yet entirely clear how CFAs are taken up by living organisms. The bioconcentration factor of CFAs has been reported to be low (Craig et al., 1990). Even if high concentrations of CFAs have been found in sediments in areas close to pulp and paper mills using chlorine bleaching (Leach & Thakore, 1977; Easty et al., 1978; Voss & Rapsomatiotis, 1985; Remberger et al., 1990) and high concentrations of CFAs have been found in the fish from such areas (Håkansson et al., 1991; Wesén et al., 1992; Mu, 1996), there is no evidence that CFAs can be taken up in fish by absorption through skin or gills. As reported by Lehtinen et al. (1991) when exposing the solid fraction of bleached kraft mill effluents (BKME) to rainbow trout in the laboratory, the fish did not show an elevated EOC1 content compared with control fish.

It is most likely, however, that the main part of CFAs, at least in animals with high concentrations of CFAs, is taken up via the food web. Experiments with rats have shown that CFAs as free fatty acids or bound in glycerolipids can be assimilated from food (Cunningham & Lawrence, 1976, 1977abc; Cunningham, 1980; Conacher et al., 1984; Paper V). The uptake of EOC1 and CFAs from the food has been observed also in fish (Håkansson et al., 1991; Ewald et al., 1996; Björn, 1999). Lehtinen and co-workers (1991) found that sticklebacks showed elevated concentrations of EOC1 when exposed to BKME via food.

CFAs given via the food to perch (*Perca fluviatilis*) have been found to be incorporated similarly to non-chlorinated fatty acids in complex lipids (Ewald et al., 1996). Also, no obstacles against the assimilation of CFAs have been observed in goldfish (*Carassius auratus*) and pike (*Esox lucius*) (Björn, 1999). In salmon (*Oncorhynchus nerka*), CFAs are not discriminated against during the transfer of lipids to developing roe (Mu, 1996). In studies on rats it has been observed that assimilated, food-derived CFAs and their metabolites can be transferred to the offspring via placenta and milk (Cunningham & Lawrence, 1977c; Cunningham, 1980; Conacher et al., 1984). Dichlorostearic acid, when present in food, was found in different organs of rats (Paper V). In studies on rats it has been found that, in relation to oleic acid, a relatively higher percentage of the administered dichlorostearic acid is taken up in heart tissue, while oleic acid dominates in other organs studied (Cunningham & Lawrence, 1977bc). However, CFAs were taken up by the animals to a lesser extent than the unsaturated analogues (Cunningham & Lawrence, 1977ab). On the other hand, no significant discrimination has been observed with respect to dichlorostearic acid in comparison to a non-chlorinated fatty acid in an assimilation experiment with perch (Ewald et al., 1996). Also

Björn (1999) has reported that dichlorostearic acid was taken up to a similar extent as oleic acid by fish and chironomid larvae.

Metabolism of CFAs

CFAs may undergo the same metabolism by β -oxidation as other fatty acids. For rats and human cells, this is proved by finding metabolites of dichlorostearic acid that differ by two and four methylene units (dichloropalmitic and dichloromyristic acids, respectively) from the parent molecule (Conacher et al., 1984; Gustafson-Svärd et al., 1999; Paper V). Similar findings have been reported also for brominated fatty acids (Jones et al., 1983b; Lawrence et al., 1984). However, in laboratory experiments no metabolites of halogenated fatty acids shorter than halogenated myristic acid were found (Jones et al., 1983b; Lawrence et al., 1984; Conacher et al., 1984; Gustafson-Svärd et al., 1999). Dichlorotridecanoic acid which has been found in the muscle lipids of eel from a polluted area (Björn et al., 1998b) and in bile of the eel (Martinsen et al., 1993) might have been formed outside the organisms, such as in pulp bleaching processes or possibly in combination with microbial processes in sediments and after that taken up by fish.

Mohamed and co-workers (1980) reported that 9,10-dibromopalmitic acid was not oxidised by the β -oxidation system of mitochondria. Therefore, it was suggested that the peroxisomal β -oxidation, which is directed towards chain-shortening and elimination of otherwise poorly metabolised compounds, such as very long, trans-unsaturated fatty acids or fatty acids with bulky substituents (Osmundsen et al., 1991), might metabolise brominated and chlorinated fatty acids to some extent (Jones et al., 1983ab; Björn, 1999). It has been speculated that the catabolism of halogenated fatty acids by β -oxidation might be hindered due to bulkiness of halogen atoms when these comes too close to the carboxylic moiety of the molecule (Ewald & Sundin, 1993).

However, in the liver lipids of rats, exposed to dichlorostearic acid, metabolites with shorter retention time in GC/ELCD studies than that of dichloromyristic acid (Figure 3; Paper V) indicate a metabolic pathway that can produce such compounds. It has previously been found that rats can metabolise CFAs and excrete chloride ions or water-soluble metabolites via the urine (Cunningham & Lawrence, 1976, 1977a). Cunningham & Lawrence (1976) proposed dechlorination followed by normal β -oxidation as a possible elimination pathway of CFAs. Dehalogenation of some halogen-containing fatty acids by certain microorganisms has been reported by Omori and Alexander (1978) and Weightman et al. (1985). Curragh et al. (1994) have found that ω -chlorinated fatty acids undergo β -oxidation until 4-chlorobutyric acid is formed. After that this acid is chemically lactonized to γ -butyrolactone. Also Kohler-Staube & Kohler (1989) have reported on microbial degradation of some short-chain chlorinated acids. Thus, also halogenated myristic acids may possibly undergo a

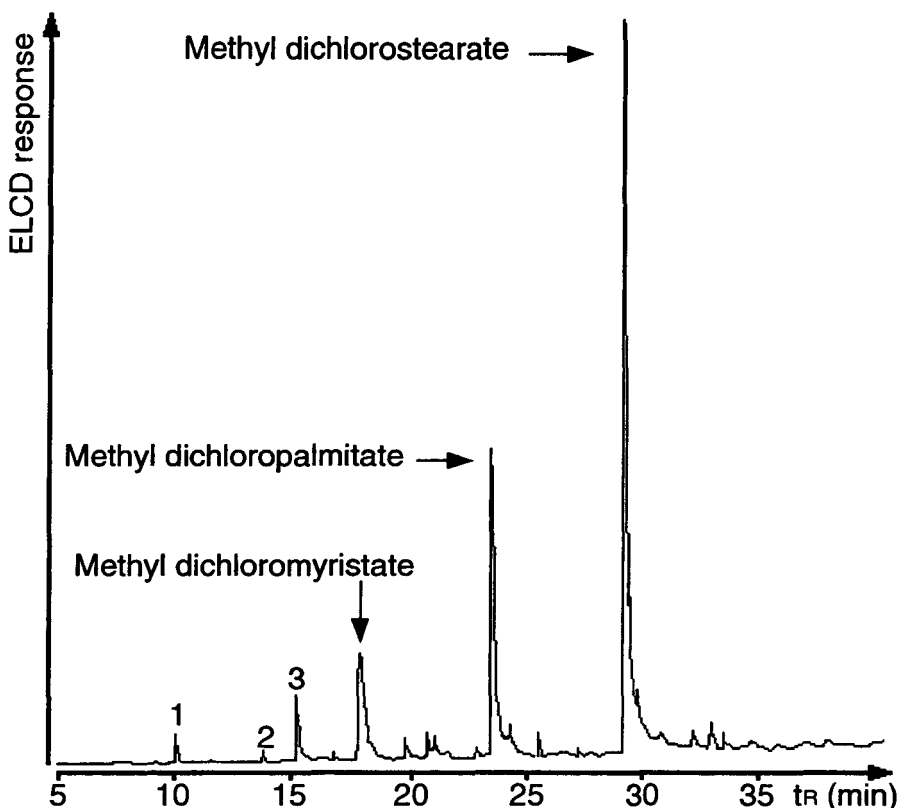


Fig. 3. ELCD chromatogram of chlorinated FAMES enriched from lipids of S9 fraction obtained from liver of rat fed a diet containing 9,10-dichlorostearic acid. The numbers indicate peaks that possibly correspond to methyl esters of metabolites of dichlorostearic acid shorter than 5,6-dichloromyristic acid. Other minor peaks represent chlorinated compounds from Arochlor 1254, which was used to induce the detoxification enzymes in the rats.

similar transformation leading to chlorinated and non-halogenated metabolites with shorter chain length.

The increase in concentrations of CFAs in roe and muscle phospholipids of migrating salmon (*Oncorhynchus nerka*) while CFAs in muscle glycerolipids were released to the same extent as non-chlorinated ones (Mu, 1996) indicates a lower turnover of CFAs in phospholipids. The dominance of dichloromyristic acid in certain samples from unpolluted areas (Wesén, 1995; Mu et al., 1996b; Milley et al., 1997) may indicate some "biological stability" of these acids. A lower turnover and higher "biological stability" in transfer through the food-chain has been found for dichlorostearic acid in comparison with oleic acid (Björn, 1999).

Biological effects of chlorinated fatty acids

A number of different adverse effects of CFAs to living organisms have been observed, several of which concern reproduction related processes. When compounds found in bleached kraft mill effluents were studied for the effects on the fertili-sation rate and sperm motility of sea urchin sperm cells, dichlorostearic acid was found to be the most toxic compound (Cherr et al., 1987). A decreasing amount of roe as well as a decreasing hatching frequency of the roe were found when zebrafish (*Brachydanio rerio*) were fed a diet rich in CFAs (Håkansson et al., 1991). Fatty acids with a high chlorine content caused a significant reduction of the arachidonic acid stimulated testosterone production in goldfish testes (Björn et al., 1998a).

Cell specific growth modulation of chlorinated oleic acid and its interaction with vitamin E and albumin has been reported (Hostmark et al., 1998). Some sublethal effects of CFAs, such as decreased growth and increased weight of certain organs have been observed for rats fed a diet containing CFAs (Cunningham & Lawrence, 1977c; Cunningham, 1980; Paper V). However, no such effects were observed by Fisher et al. (1983) in long term feeding studies of rats with cake made from chlorinated flour. In other studies, female but not male mice showed higher mortality when fed a diet containing CFAs (Neal, 1980; Ginocchio et al., 1983). The acute toxicity of dichlorostearic acid has been found to be low to rat (Cunningham, 1980) but high to fish (Leach & Thakore, 1977). No mutagenicity of lipids rich in CFAs and dichlorostearic acid itself has been found using Ames test (Håkansson et al., 1991; Paper IV). However, both increased and decreased ability to activate indirectly acting mutagens was observed in *in vitro* and *in vivo* studies of dichlorostearic acid (Paper IV, V).

The possibility has been discussed that biological effects of CFAs are caused by disturbances of membrane properties (Ewald & Sundin, 1993) after incorporation of CFAs into cell membranes (Björn et al., 1998b) or caused by CFAs affecting the composition of fatty acids in membrane lipids (Paper V). The changes in the ratio between saturated and unsaturated fatty acids in cell membranes determine membrane fluidity and permeability (Gurr & Harwood, 1991). Although CFAs may have configurations with some similarity to unsaturated ones (Ewald & Sundin, 1993) they are saturated fatty acids. Therefore, the less efficient regulation of membrane fluidity may arise when CFAs are incorporated in cell membranes (Ewald, 1999). Also, due to slower metabolism of CFAs, if they are present in high concentrations (in highly polluted areas up to one percent of total fatty acids in fish can be chlorinated; Håkansson et al. (1991)), CFAs may contribute to that an organism is supplied with less energy (Ewald, 1999).

No induction of Cytochrome P450 enzymes by CFAs was found by Goksøyr & Larsen (1993). Also *in vivo* tests measuring EROD-activity of lipids rich in CFAs

failed to give a response (Håkansson et al., 1991). This and also an uptake and distribution more or less similar to that of the common, non-chlorinated fatty acids of CFAs within different organisms indicates that CFAs are not identified by the organism as xenobiotic compounds. The properties similar to those of ordinary, non-chlorinated fatty acids in combination with some toxicity can make elevated concentrations of CFAs a possible problem for the organisms exposed to them.

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