

# **Determination of chlorinated fatty acids using SPE, XSD and GC/MS**

**with particular regard to cultured human cells**

**Gunilla Åkesson Nilsson**

*Department of Environmental Assessment  
Uppsala*

**Doctoral thesis  
Swedish University of Agricultural Sciences  
Uppsala 2004**

**Acta Universitatis Agriculturae Sueciae**  
Agraria 493

ISSN 1401-6249  
ISBN 91-576-6773-X  
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Tryck: SLU Service/Repro, Uppsala 2004

*Lycka är en biprodukt som utvinnes  
vid framställningen av något annat.*

*Aldous Huxley*

*Man bör gripa sig an en lätt sak  
som om den vore svår och en  
svår sak som om den vore lätt.*

*Baltasar Gracián*

ur 301 ordspråk och visdomsord  
av Leif Eriksson och Kristoffer Lind,  
Lind & Co 2004



# Abstract

Åkesson-Nilsson, G. 2004. *Determination of chlorinated fatty acids using SPE, XSD and GC/MS with particular regard to cultured human cells*. Doctor's dissertation.

ISSN 1401-6249, ISBN 91-576-6773-X

Chlorinated fatty acids (CIFAs) account for a considerable portion of the extractable, organically bound chlorine (EOCl) in aquatic animals. Because of analytical difficulties little is as of yet known about their behaviour in organisms. Some of the analytical problems were solved by using a recently introduced halogen specific detector (XSD) for gas chromatography. It was shown that the XSD is a good alternative to the previously used electrolytic conductivity detector to detect CIFA methyl esters (CIFAMEs). Furthermore, the XSD was found to be very easy to maintain and stable in the analysis of CIFAMEs. The XSD operates in an oxidative pyrolysis mode and the sample compounds are converted into their oxidation products. When halogen-containing compounds enter the hot detector, the detector current will increase.

The incorporation and metabolism of dichlorooctadecanoic acid in a cell-lines of human cells were studied. The acid was incorporated and degraded to dichlorohexadecanoic acid and dichlorotetradecanoic acid by  $\beta$ -oxidation. CIFAs were found both in the neutral lipid and in the phospholipid fractions of the cultured cells. No shorter CIFAs than dichlorotetradecanoic acid were detected indicating that no further metabolism occurred. Dichlorotetradecanoic acid was released from the cells into the culture medium to a higher extent than were the other CIFAs.

An isolation method for CIFAMEs was developed in order to separate the metabolite dichlorotetradecanoic acid from vastly dominating common unchlorinated fatty acids. By using solid-phase extraction on aminopropyl silica > 1  $\mu$ g of a CIFAMEs can be isolated and detected from 1 g of lipid with only 1% of the dominating unchlorinated FAMEs in the fraction containing the CIFAMEs.

Structure elucidation of CIFAs was done with gas chromatography/mass spectrometry using picolinyl esters of the CIFAs and electron ionization. The picolinyl esters made it possible to identify 5,6-dichlorotetradecanoic acid as a metabolite of 9,10-dichlorooctadecanoic acid, which further supports that Cl<sub>2</sub>FAs are to an extent degraded by  $\beta$ -oxidation. Methyl esters or pyrrolidides of CIFAs are not suitable for full characterisation of Cl<sub>2</sub>FAs, such as localisation of the chlorine atoms.

*Keywords:* mass spectrometry, halogen sensitive detector, organohalogens, triacylglycerols, dichlorostearic acid, dichloromyristic acid, metabolism, membrane

*Author's address:* Gunilla Åkesson Nilsson, Department of Chemistry and Biomedical Sciences, Kalmar University, SE-391 82 Kalmar, Sweden and Department of Environmental Assessment, Swedish University of Agricultural Sciences, P.O. Box 7050, SE-750 07 Uppsala, Sweden. [gunilla.akesson-nilsson@hik.se](mailto:gunilla.akesson-nilsson@hik.se)

# Populärvetenskaplig svensk sammanfattning

Denna avhandling handlar om bestämning av klorerade fettsyror. De klorerade fettsyrorerna är intressanta föreningar eftersom de visar sig kunna utgöra en stor del av de fettlösliga klororganiska föreningarna i djur som lever i vatten, tex fisk, hummer, musslor, tumlare och säl. De välkända klororganiska miljögifterna som PCB, DDT och dioxiner utgör vanligen endast en liten del, mycket mindre än 5%, av de fettlösliga klororganiska föreningarna, medan klorerade fettsyror ofta utgör en betydligt större andel. Merparten av de klorerade fettsyrorerna som man har funnit i fisk kommer troligen från fabriker som producerar klorblekt pappersmassa. Klorerade fettsyror har även påträffats i en del kakor och kex, vilket beror på att mjölet som används för dessa bakverk har klorerats för att få särskilda bagegenskaper. Det har även visat sig att klorerade fettsyror kan bildas i samband med desinficering av livsmedel med klor, tex kött, men även i vatten, om det förekommer rester av omättat fett i vattnet. Det är de omättade fettsyrorerna som reagerar med klor och bildar klorerade fettsyror.

Många klororganiska föreningar klassas som miljögifter eftersom de kan lagras i fettvävnaden och på så vis på sikt uppnå halter som kan ge negativa effekter på den enskilda organismen. Det är inte känt om klorerade fettsyror förekommer i människor, men det är troligt eftersom både  $\omega$ -3-fettsyror, typiska för fiskfett, och PCB förekommer i högre halter hos människor som äter mycket fisk än hos dem som äter lite fisk.

För att kunna bestämma klorerade fettsyror i miljöprover krävs det <sup>i)</sup> en detektor som är speciellt känslig för klororganiska föreningar, <sup>ii)</sup> en omfattande uppberedning av provet, för att få bort de ointressanta föreningarna i ett prov så merparten av provet som återstår består av de klorerade fettsyrorerna och <sup>iii)</sup> en teknik som kan ge information om strukturen på de ingående klorerade fettsyrorerna.

Gaskromatografi (GC) är en vanlig teknik för att analysera organiska föreningar. Principen är att provet, som kan innehålla många olika föreningar, förångas och sedan transporteras med en bärgas genom en kolonn. Beroende på olika egenskaper hos föreningarna i provet så tar det olika lång tid för dem att passera kolonnen och på så vis separeras föreningarna. När en förening lämnar kolonnen passerar den en detektor som registrerar att föreningen passerar.

Då de klorerade fettsyrorerna är bundna i fett och i cellmembran (skyddsbarriären mellan cellens innehåll och omvärlden utanför) så måste de först omvandlas till enklare föreningar som lämpar sig bättre för separation med gaskromatografi. Fettsyrametylestrar är derivat av fettsyror, som lämpar sig bra för gaskromatografi.

Då de klorerade fettsyrorerna förekommer i mycket låga halter, i jämförelse med de vanliga oklorerade fettsyrorerna, som förekommer i flera 1000 gånger högre halter än de klorerade, så krävs det en detektor som är specifik för klorerade föreningar. Nyligen introducerades en ny detektor som är känslig för klororganiska föreningar, XSD, som jämfört med andra likvärdiga klorkänsliga detektorer är enklare att använda och ger en stabilare signal. Vi var de första som använde XSD för att analysera klorerade fettsyrorerna vilket beskrivs i uppsats I.

Det största problemet med att isolera de klorerade fettsyror är att separera dem från de vanliga fettsyrorna. I uppsats II beskrivs en enkel metod för att separera klorerade från oklorerade fettsyror. Metoden baseras på fastfasextraktion, vilket innebär att föreningarna i provextraktet separeras på en liten kolonn av ett material som binder de klorerade fettsyrorna, men inte de oklorerade. När större delen, 99 %, av de oklorerade fettsyrorna tvättats ur kolonnen kan man få fram en lösning av de klorerade fettsyrorna med bara en liten mängd oklorerade fettsyror kvar. Med hjälp av denna teknik och en XSD är det möjligt att detektera en miljontedels gram klorerade fettsyror i ett gram fett.

Masspektrometri är en teknik som kan ge information om strukturen hos en kemisk förening. I masspektrometern slås de i provet ingående föreningarna sönder i karaktäristiska fragment som kan registreras som ett masspektrum som är mer eller mindre specifikt för varje ämne; varje föreningen har då sitt eget "fingeravtryck". Man kan även tolka masspektret från ett okänt ämne för att ta reda på vilken struktur ämnet har. I Uppsats III arbetade vi med att ta reda på var på kolledjan kloratomerna sitter i en okänd klorerad fettsyra. Eftersom fettsyrorna i sig själva inte går att få igenom en gaskromatograf måste de först omvandlas, derivatiseras, till något som är möjligt att få igenom gaskromatografen. Vi testade tre olika typer av derivat. Det visade sig att bästa möjliga information om strukturen hos klorerade fettsyror får man om de studeras som picolinylestrar.

Det är svårt att avgöra om klorerade fettsyror även finns i människor eftersom det är svårt att få tillgång till vävnad från människa på grund av smittorisk och av etiska skäl. Eftersom proverna därmed ofta är små är det viktigt att det finns bra metoder med vilka man kan bestämma klorerade fettsyror i låga halter. En annan viktig väg att ta reda på om klorerade fettsyror utgör ett problem för människan är att studera om celler från människa kan ta upp klorerade fettsyror. I uppsats IV visade vi att celler från människa som odlas utanför människokroppen kan ta upp klorerade fettsyror och lagra dem i sitt fett och troligen även i sina cellmembraner. Dessutom visade vi att dessa celler kan bryta ner, de klorerade fettsyrorna till kortare klorerade fettsyror.

Processen med inbindning och nerbrytning av ett ämne kallas för metabolism och de kortare klorerade fettsyrorna är i det här fallet metaboliter av de längre klorerade fettsyrorna.

Genom att använda isoleringsmetoden som beskrivs i uppsats II gick det att isolera en av metaboliterna som bildas när en klorerad fettsyra bryts ner i cellkulturen av mänskliga celler. Med hjälp av masspektrometrimetoden som beskrivs i uppsats III var det sedan möjligt att bestämma strukturen för den isolerade metaboliten. Med hjälp av denna struktur så stärktes bevisen för att klorerade fettsyror kan brytas ner på liknande sätt som de som vanliga oklorerade fettsyrorna.



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# Preface

## Paper I-IV

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

- I. Åkesson-Nilsson G., Nilsson O., Odenbrand I., Wesén C. 2001. New halogen-specific detector applied to the analysis of chlorinated fatty acids. *Journal of Chromatography A* 912:99-106.
- II. Åkesson-Nilsson G. 2003. Isolation of chlorinated fatty acid methyl esters derived from cell-culture medium and from fish lipids by using an aminopropyl solid-phase extraction column. *Journal of Chromatography A* 996:173-180.
- III. Åkesson-Nilsson G., Wesén C. 2004. Structural characterisation of 5,6-dichlorotetradecanoic acid, an isolated metabolite of 9,10-dichlorooctadecanoic acid, by studying picolinyl esters, pyrrolidides and methyl esters with electron ionisation mass spectrometry. *Journal of Mass Spectrometry* 39:1313-1320.
- IV. Gustafson-Svärd, C., Åkesson-Nilsson, G., Mattsson, M., Sundin, P., Wesén, C. 2001. Removal of xenobiotic dichlorostearic acid from phospholipids and neutral lipids in cultured human cell lines by  $\beta$ -oxidation and secretion of dichloromyristic acid. *Pharmacology & Toxicology* 89:56-64.

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## Abbreviations

<b>amu</b>	atomic mass unit
<b>C<sub>16:0</sub></b>	hexadecanoic acid
<b>C<sub>18:0</sub></b>	stearic acid, octadecanoic acid
<b>C<sub>18:1</sub></b>	oleic acid, octadecenoic acid
<b>C<sub>18:2</sub></b>	linoleic acid, octadecadienoic acid
<b>C<sub>18:3</sub></b>	linolenic acid, octadecatrienoic acid
<b>C<sub>20:4</sub></b>	arachidonic acid, eicosatetraenoic acid
<b>CI</b>	chemical ionisation
<b>Cl/H</b>	chlorine/hydrocarbon ratio
<b>Cl<sub>2</sub>C<sub>14</sub></b>	dichlorotetradecanoic acid
<b>Cl<sub>2</sub>C<sub>16</sub></b>	dichlorohexadecanoic acid
<b>Cl<sub>2</sub>C<sub>18</sub></b>	dichlorooctadecanoic acid
<b>Cl<sub>2</sub>FA</b>	dichlorinated fatty acid
<b>CIFAME</b>	chlorinated fatty acid methyl ester
<b>CIFA</b>	chlorinated fatty acid
<b>CIOHFA</b>	chlorohydroxy fatty acid
<b>DDT</b>	trichloro-2,2-bis(4-chlorophenyl)ethane
<b>DHA</b>	docosahexaenoic acid
<b>DMOX</b>	4,4-dimethyloxazoline
<b>ECD</b>	electron capture detector
<b>EI</b>	electron ionisation
<b>ELCD</b>	electrolytic conductivity detector
<b>EOCI</b>	extractable, organically bound chlorine
<b>EPA</b>	eicosapentaenoic acid
<b>FAME</b>	fatty acid methyl ester
<b>GC</b>	gas chromatography
<b>HOCl</b>	hypochlorous acid
<b>HPLC</b>	high performance liquid chromatography
<b>INT 407</b>	Intestine 407
<b>m/z</b>	mass/charge ratio
<b>MS</b>	mass spectrometry
<b>PCB</b>	polychlorinated biphenyl
<b>PUFAME</b>	polyunsaturated fatty acid methyl ester
<b>SIM</b>	selective ion monitoring
<b>SPE</b>	solid-phase extraction
<b>TIC</b>	total ion current
<b>TLC</b>	thin layer chromatography
<b>XSD</b>	halogen sensitive detector
<b>ω-3-FA</b>	ω-3-fatty acid

## Objectives

- To develop simple methods for isolation and characterisation of chlorinated fatty acids (CIFAs) in samples dominated by common unchlorinated fatty acids and containing low concentrations of extractable organically bound chlorine (EOCl),  $\ll 30 \mu\text{g/g}$  lipid. The detection, isolation and characterisation of CIFAs were focused on techniques such as halogen specific detection (an XSD connected to a gas chromatograph), solid-phase extraction (SPE), and mass spectrometry (MS).
- To study the incorporation, metabolism and distribution of CIFAs in human cell lines cultured in a medium containing *threo*-9,10-dichlorooctadecanoic acid (9,10-Cl<sub>2</sub>C<sub>18</sub>).

## Introduction

### Background

CIFAs have recently attracted attention as they have been found to constitute up to 90% of EOCl in aquatic animals (Wesén, Carlberg & Martinsen 1990; Wesén *et al.* 1992a; Wesén 1995; Wesén *et al.* 1995a; Mu 1996; Milley *et al.* 1997). Well-known chlorinated pollutants such as polychlorinated biphenyls (PCB), dioxins, and chlorinated pesticides normally contribute to only a minor portion (<5%) of EOCl in aquatic species (Lunde, Gether & Steinnes 1976; Södergren *et al.* 1988; Newsome *et al.* 1993).

Several forms and isomers of CIFAs are mentioned in the literature (White & Hager 1977; Wesén *et al.* 1992b; Mu *et al.* 1996a; Björn *et al.* 1998; Vereskuns 1999; Dembitsky & Srebnik 2002), but long-chain vicinally dichlorinated fatty acids (Cl<sub>2</sub>FAs) (the chlorine atoms are bound to adjacent carbon atoms) (Figure 1) are those most commonly reported (Wesén *et al.* 1992b; Mu *et al.* 1996a; Björn *et al.* 1998a; Vereskuns 1999; Zhuang *et al.* 2003a). Cl<sub>2</sub>FAs have been connected to anthropogenic inputs such as effluents from chlorine bleached pulp production (Wesén *et al.* 1992a; Wesén 1995; Björn *et al.* 1998), which is a major source of Cl<sub>2</sub>FAs (Leach & Thakore 1977). Chlorohydroxy fatty acids (ClOHFAs) are another group of CIFAs that possibly can make up a considerable portion of CIFAs (White & Hager 1977; Björn 1999; Vereskuns 1999; Clark 2003). In the ClOHFA the chlorine atom and the hydroxyl group are also bound to adjacent carbon atoms.

The distribution of CIFAs is not well known, but they have so far been found in fish in Scandinavian, Baltic, Alaskan, and Ontario waters (Wesén *et al.* 1995a; Mu 1996; Björn *et al.* 1998; Vereskuns 1999; Zhuang *et al.* 2003a; Mu *et al.* 2004; Zhuang *et al.* 2004), bivalves from Scandinavian waters (Wesén *et al.* 1995b),

lobster from Canadian east-coast (Milley *et al.* 1997), and marine mammals from the Swedish west-coast (Clark, 2003), but they can most probably also be found in other water areas. ClFAs have also been detected in several foodstuffs, in the USA (KAN-DO Office & Pesticides Team 1995), probably originating from chlorinated flour (Komo-Suwelack, Schulte & Acker 1983; Fukayama *et al.* 1986; Heikes 1992, 1993).

Little is known of the toxicity of ClFAs. Only a few studies of physiological effects have been reported, and they relate particularly to effects on reproduction related processes such as reduced sea urchin sperm cells motility (Cherr *et al.* 1987); reduction in egg hatching frequency for zebrafish (Håkansson *et al.* 1991); and, reduction of the arachidonic acid-stimulated testosterone production in goldfish testes (Björn, Sundin & Wesén 1998). Also more general adverse effects such as impaired cell-growth and elevated mortality of fish have been suggested (Leach & Thakore 1977; Høstmark *et al.* 1998; Høstmark *et al.* 1999). On the cellular level, increased release of ATP has been observed in cells exposed to Cl<sub>2</sub>C<sub>18</sub> (Ewald & Sundin 1993).

The ClOHFAs have been shown to cause physiological effects such as ATP depletion in human cells (Dever *et al.* 2003). Furthermore, they have been shown to cause cell death in human vein endothelial cells (Visser, Carr & Winterbourn 2001).

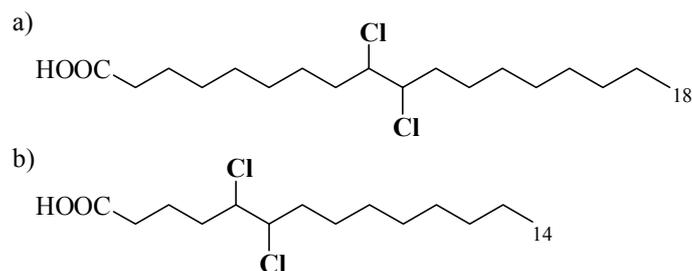


Figure 1. Vicinal a) 9,10-dichlorooctadecanoic acid (9,10-Cl<sub>2</sub>C<sub>18</sub>) and b) 5,6-dichlorotetradecanoic acid (5,6-Cl<sub>2</sub>C<sub>14</sub>).

This thesis is primarily focused on the determination of the most commonly found Cl<sub>2</sub>FAs, such as 9,10-Cl<sub>2</sub>C<sub>18</sub> and dichlorotetradecanoic acid (Cl<sub>2</sub>C<sub>14</sub>) (Figure 1). 9,10-Cl<sub>2</sub>C<sub>18</sub> is likely formed by chlorination of the unsaturated oleic acid (C<sub>18:1</sub>) present in wood extracts (Leach & Thakore 1977), but also in flour (Komo-Suwelack, Schulte & Acker 1983; Heikes 1993). Cl<sub>2</sub>C<sub>14</sub> is suggested to be a metabolite of Cl<sub>2</sub>C<sub>18</sub> (Conacher *et al.* 1984; Wesén 1995; Björn 1999). The possible biogenic origin of ClFAs has not been closely studied, but ClOHFAs are suggested to be formed in the mammalian cellular defence system. During infections several types of cells produce hypochlorous acid (HOCl), molecular chlorine, or hypochlorite which possibly may react with unsaturated fatty acids and form ClOHFAs (Winterbourn *et al.* 1992; Winterton 1997; Arnhold *et al.* 2002; Spalteholz *et al.* 2004).

The CIFAs have been found to be incorporated in storage lipids (triacylglycerols) and in membrane lipids (phospholipids) (Figure 2) in all tissues studied (including brain) (Ewald *et al.* 1996; Björn *et al.* 1998; Vereskuns 1999). CIFAs possess a certain stability and can be actively accumulated by organisms (Björn 1999; Ewald 1999).

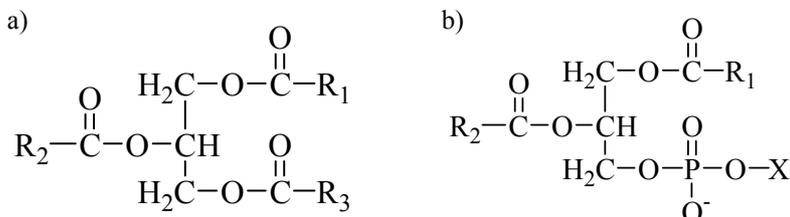


Figure 2. Lipids in which the CIFAs are bound to a) storage lipids (triacylglycerols) b) membrane lipids (phospholipids) X is derived from an alcohol.

### Determination of chlorinated fatty acids

Determination methods for trace amounts of CIFAs have been developed relatively recently, which may explain why these compounds did not come to the attention of environmental scientists until in the 1990s. The analysis of CIFAs usually involves extraction of the lipids, transesterification of the fatty acids from glycerol esters to methyl esters followed by separation by gas chromatography (GC) and detection by a halogen sensitive detector, or by MS, which also allows identification. Fatty acid methyl esters (FAMES) are common derivatives of fatty acids in GC-analysis (Figure 3).

An important and problematic step in the analysis of CIFAs is the removal of unchlorinated lipids. Methods for the separation of CIFAs from unchlorinated fatty acids, and their corresponding CIFAMES and FAMES, are, *e.g.*, silver ion and urea complexation (Mu *et al.* 1996b), thin layer chromatography (TLC) (Mu *et al.* 1996b), and gel permeation chromatography (Sephadex LH-20) (Milley *et al.* 1997). These methods are only suitable for EOC1 concentrations >30 µg/g lipid and they are generally laborious. Other approaches such as cold trapping of GC eluate (Wesén *et al.* 1995a) and high performance liquid chromatography (HPLC) (Zhuang *et al.* 2003b) have also been used to isolate CIFAMES.

Another challenge in the determination of the CIFAs is the detection. The electron capture detector (ECD) most commonly used for traditional organohalogen contaminants is not suitable for detection of CIFAMES (Wesén *et al.* 1992b). Instead, the electrolytic conductivity detector (ELCD) has been found to be very useful to determine CIFAs (Wesén *et al.* 1992b). However, this detector is difficult to operate and maintain. MS in the selected ion monitoring (SIM) mode can also be useful to detect CIFAs (Sundin *et al.* 1992) by acquiring and recording the ion current only at certain selected mass/charge (*m/z*) values. This technique can exclude monitoring of co-eluted common unchlorinated FAMES, but at the same time only known compounds are monitored, and unknown CIFAs may remain undetected.



MS has become an important chemical analytical tool in CIFA identification. The principle of MS is based on <sup>i)</sup> ionisation of an analyte in an ion source <sup>ii)</sup> separation of the created charged particles according to their  $m/z$  ratio in an analyser and <sup>iii)</sup> measurement of the relative abundance of ionised species of each fragment in a data handling system. A more or less unique mass spectrum is generated that may be used as a chemical “fingerprint” to characterise a compound. The fragment ions provide information on the structure of the mother compound. Using multiple ionisation methods, such as electron ionisation (EI) at 70 eV and chemical ionisation (CI) gives additional information on the molecular structure of a compound. To ascertain the molecular weight of CIFAs CI is the better choice (Sundin *et al.* 1992; Zhuang *et al.* 2004). CI with ammonia as the reagent gas is a soft ionisation method that produces abundant adduct ions of the intact CIFAME molecule. EI of CIFAMEs often result in loss of chlorine atoms, and the intensity of chlorine containing fragment ions are therefore very low or absent in their EI mass spectra (Sundin *et al.* 1992; Wesén *et al.* 1995a; Mu *et al.* 1996a). Furthermore, fragments of a coeluting enoic FAME are observed in these spectra. Structural information such as the position of the chlorine atoms cannot be determined by EIMS or ammonia CIMS of CIFAMEs. The chlorine atoms in Cl<sub>2</sub>FAMEs are largely lost when utilising MS in the EI mode, and ammonia CI results in a molecular adduct ion, with no clues to the positions of the Cl atoms.

To study the location of Cl in the carbon chain different derivatives such as pyrrolidides and 4,4-dimethyloxazoline (DMOX) of the Cl<sub>2</sub>FAs have been used (Åkesson-Nilsson 1996; Zhuang, Mc Kague & Reeve 2004). These derivatives are commonly used for localisation of double bonds in unsaturated fatty acids (Andersson & Holman 1974; Christie *et al.* 2000; Dobson & Christie 2002). The DMOX derivative of Cl<sub>2</sub>FAs is more suited for determining the position of the chlorine atoms than the pyrrolidides, because the DMOX derivative generates fragments that are characteristic for positional isomers (Zhuang, Mc Kague & Reeve 2004). By using the pyrrolidides derivatives, the position of the chlorine atoms on the carbon chain can only approximately be determined (Åkesson-Nilsson 1996). However, as the intensity of the fragments in the high mass range of the pyrrolidides is higher than for the corresponding DMOX derivative of Cl<sub>2</sub>FA, the pyrrolidides should be the choice when the molecular weight of a long-chained Cl<sub>2</sub>FA should be suggested.

### **Expected levels of chlorinated fatty acids in human tissues**

The presence of CIFAs in human tissue has not yet been reported, but it is anticipated, as typical  $\omega$ -3-fatty acids ( $\omega$ -3-FAs) from fish can be found in humans who consume fish (Bjerregaard *et al.* 2003). The daily intake of typical fish  $\omega$ -3-FAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) depends on which species of fish is consumed, because different fish species differ substantially in the  $\omega$ -3-FAs content from about 0.1 mg to about 40 mg/g fresh weight of fish (Mahaffey 2004). Eating 100 g fish per day containing about 10 mg  $\omega$ -3-FAs/g fish (Mahaffey 2004), would correspond to an intake of about 1 g  $\omega$ -3-FAs per day. The daily intake of CIFAs in human is more difficult to estimate, but <sup>i)</sup> if the fat content of the fish is 5%, and <sup>ii)</sup> provided that the content of EOCl is 50 mg EOCl/kg lipid, and <sup>iii)</sup> that the CIFAs make up to about 50% of the EOCl, and

iv) that the average molecular weight is assumed to be 300 g/mol, the daily intake of ClFAs would be about 1 mg per day, corresponding to 1/1000 of the intake of  $\omega$ -3-FAs.

The content of  $\omega$ -3-FAs in blood serum from individuals in Japan was found to be about 5 and 15% of the lipids in individuals assumed to eat approximately 90 and 200 g fish, respectively, per day (Yamada *et al.* 2000). Judging from these levels of  $\omega$ -3-FAs in blood serum, the ClFAs might possible contribute to about 0.005-0.015% (50-150  $\mu$ g/g lipid) of the lipids. In muscle phospholipids from a harbour porpoise (*Phocaena phocaena*) a marine mammal, the ClFAs were estimated to contribute to up to 6 mg Cl/g lipid, corresponding to 3-6% of the lipids being chlorinated (Clark 2003). The expected levels of ClFAs in humans in Sweden is probably much lower than in the porpoise and in those who hypothetically consume 100 g fish per day, because Swedes only eat about 200 g of fish per person per week (Bernes 1998) and much of this fish is caught in waters with less pollutant load than the Baltic. However, even if the level of the ClFAs seems to be very low compared to the common unchlorinated fatty acids the estimated content of ClFAs may be of possible concern.

The studies summarised in this thesis address the following questions:

- i) Is there a halogen sensitive detector that meets the demands of trace analysis of Cl<sub>2</sub>FAs in multiple samples? (Paper I)
- ii) Is it possible to find a simple and efficient method to isolate Cl<sub>2</sub>FAs from common unchlorinated fatty acids? (Paper II)
- iii) Is there a derivative of Cl<sub>2</sub>FAs that will allow determination of the location of the chlorine atoms on the carbon chain and the molecular weight of Cl<sub>2</sub>FAs by EIMS? (Paper III)
- iv) Can human cells incorporate and metabolise Cl<sub>2</sub>FAs? (Paper IV)
- v) Is it possible to isolate a metabolite of a Cl<sub>2</sub>FA and determine its metabolic origin? (Paper III)

## A halogen sensitive detector (XSD)

Recently, a new halogen specific detector (XSD) for gas chromatography was introduced for the determination of halogenated compounds, such as chlorinated pesticides and PCBs (Anonymous 1998a; Cook & Engel 1999). The XSD is comparable to the ELCD with regards to the detection limit of such compounds, and it has several advantages over the ECD and the ELCD as a halogen detector (Anonymous 1998a). It is easier to maintain, and it has a higher selectivity (expressed as the chlorine/hydrocarbon ratio (Cl/H), than the ECD) (Anonymous 1995, 1998a). These properties make the XSD interesting also for the determination of ClFAs.

### The principle of the XSD

The XSD consists a temperature regulated reactor core and a probe assembly mounted inside the core. The reactor core is an anodic platinum coil, wrapped around and in contact with an alkali glass ceramic rod and a cathodic platinum bead at the end of the rod (Figure 1 in Paper I). A potential is applied over the anode and cathode and a background current is obtained, probably caused by thermal electrons emitted from the cathode at high temperatures. When halogen containing compounds enter the hot detector, the detector current will increase (Anonymous 1998a).

The alkali in the glass ceramic rod can activate the platinum cathode by decreasing the work function of the cathode from that of platinum to a value close to that of the alkali element (*i.e.* from 5.65 eV to between 2 and 3 eV (Handbook of Chemistry and Physics 1992-1993). The activation of the cathode makes the work function lower than the electron affinity of chlorine (3.6 eV) (Handbook of Chemistry and Physics 1992-1993) and an electron transfer to the chlorine can occur.

The XSD reactor operates in an oxidative pyrolysis mode and the sample compounds are converted into their oxidation products. To investigate which oxidation products might be formed during combustion of chlorine containing compounds, a chemical reaction and equilibrium software with an extensive thermochemical database was used (Paper I). -A temperature interval of 500-1500 °C was studied and a pressure of 1.0 bar was assumed. The following raw material were selected; 5 μmol of Cl<sub>2</sub> (g), 100 μmol of H<sub>2</sub>O (g), 80 mol of N<sub>2</sub> (g), and 20 mol of O<sub>2</sub> (g). Reactions with the ions H<sup>+</sup> and OH<sup>-</sup> were also simulated.

According to this theoretical chemical equilibrium study, free gaseous chlorine (Cl (g)) is the major compound formed at temperatures above 930 °C and HCl (g) is the major oxidation product in the temperature range 600-900 °C (Paper I). Cl (g) is believed to absorb on to the alkali activated cathode (Anonymous 1998a) and remove alkali from the surface of the ceramic rod close to the anode, leading to an increased detector current (Rice 1951; Roberts 1957). The process

responsible for the transfer of alkali from the ceramic rod to the cathode (the activation of the cathode) is not explained in literature.

The choice of ceramic rod material is important in order to maintain a low detection limit and a long lasting detector, because the halogen mediated removal of alkali from the surface of the ceramic rod eventually results in a lack of alkali and the ceramic rod must be replaced by a new one. What the alkali material content is in the ceramic rod in the XSD for sale is not mentioned in the operator's manual or the application, but (Anonymous 1998b) mentions sodium as the alkali metal present.

To investigate the nature of the material in the ceramic rod, a scanning electron microscope was used (Paper I). This study showed that the ceramic rod contains potassium, aluminium, and silica (Paper I), and according to Rice (1951) Leucite ( $K_2O-Al_2O_3-SiO_2$ ; 1:1:4) gives low detection limit and very long constant life time. Potassium, instead of sodium, probably makes the detector more sensitive, because potassium has a lower work function, 2.30 eV, than sodium, 2.75 eV (Handbook of Chemistry and Physics 1992-1993) causing a larger difference between the work function of the potassium activated platinum and the electron affinity of chlorine.

## **XSD in the determination of chlorinated fatty acids**

### *The XSD response*

The XSD response depends on the flow of the combustion gas (being either air or oxygen), the reactor temperature, and the amount of injected compound (Anonymous 1998a) (Paper I). To study if the XSD can be used as a detector alternative to the ELCD for the analysis of methyl esters of CIFAs, the detection limit and the selectivity in terms of the response ratio between chlorinated and unchlorinated FAMES were studied and compared to those previously obtained for ELCD (Paper I).

The XSD was connected to a GC equipped with an unpolar fused silica capillary column, and a manual wide bore injection technique (splittless) was used. For optimizing the XSD conditions different concentrations of  $Cl_2C_{18}$  methyl ester were studied at different air flows through the detector and at different detector temperatures.

The highest response of  $Cl_2C_{18}$  methyl ester was obtained at an air flow of 10-20 ml/min and at a temperature of 1100 °C. In the experiment where different air flows were studied it was found that the response of CIFAMES was constant in the flow range of 10-20 ml/min. However, the response was reduced as the air flow exceeded 20 ml/min or was below 10 ml/min (Paper I). An explanation for the reduced response at higher air flows might be that the compound will remain in the detector for a shorter time (Anonymous 1998a). The reason for the decrease in response also when the air flow is lower than 10 ml/min is not known (Paper I).

It was found that the reactor temperature is an important factor for the detector response. As the detector temperature was decreased from 1100 °C to 1000 and 900 °C the response of Cl<sub>2</sub>C<sub>18</sub> methyl ester was reduced by 50-55% and 80-85%, respectively. An explanation to the reduced response at lower temperature might be that the formation of Cl (g), supposedly the species that generates the increased current (Anonymous 1998a), decreases when temperature decreases (Paper I). However, Cl (g) is probably not the only compound that generates a current in the reactor, as the decrease of the concentration of Cl (g) with decreasing reactor temperature is not proportional to the reduced response of chlorinated test compounds (Paper I).

The detector response of Cl<sub>2</sub>C<sub>18</sub> methyl ester was linear from 0.2 to 8 ng (corresponding to about 0.04-2 ng of chlorine), but non-linear when larger amounts were injected, and lower than expected (Paper I). The non-linearity is possibly caused by an insufficient access to alkali atom-activated surfaces.

#### *The chlorine/hydrocarbon selectivity*

As common FAMES are found in the samples in great excess over the ClFAMES another important property of a halogen selective detector is the Cl/H selectivity. The selectivity in terms of the (Cl<sub>2</sub>C<sub>18</sub> methyl ester)/ (C<sub>18:1</sub> methyl ester) signal ratio was determined. The selectivity was studied at different reactor temperatures and it was found to decrease with increasing temperature (Figure 4), which indicates that at higher temperature, unchlorinated compounds generate oxidation products that also increase the detector current. The selectivity at 1100 °C was 3 x 10<sup>3</sup>, and at 1000 °C it was 10<sup>4</sup>, *i.e.*, the same order of magnitude as the Cl/H response given by the manufacturer (Anonymous 1998a). At 900 °C the selectivity was >>10<sup>4</sup> (no signal was recorded from 5 µg of injected C<sub>18:1</sub> methyl ester), which is comparable to the Cl/H selectivity (10<sup>6</sup>) of the ELCD (Farwell, Gage & Kagel 1981). This was confirmed by comparing the XSD response to the ELCD response of an eel lipid extract containing different ClFAMES that previously has been studied in depth by ELCD; (Mu *et al.* 1996a). The ClFAME pattern of these chromatograms and the relative intensities of the peaks were nearly equal (Figure 4 in Paper I).

#### *The detection limit of dichlorooctadecanoic acid methyl ester*

Thus, when using the XSD it is important to select a proper reactor temperature as the apparent advantage of performing the analyses at higher temperatures is counteracted by the reduced selectivity (Paper I). The detection limit of the Cl<sub>2</sub>C<sub>18</sub> methyl ester measured at a reactor temperature of 900 °C with an air flow of between 10 and 20 ml/min, was determined to be 0.2 ng (at two times the noise). This is similar to the detection limit in GC/ELCD (Wesén *et al.* 1992b) and not notably different from that (0.1 ng) obtained by ammonia positive ion CIMS in the SIM mode (Sundin *et al.* 1992). However, if a higher detector temperature is selected lower amounts of ClFAMES can be detected.

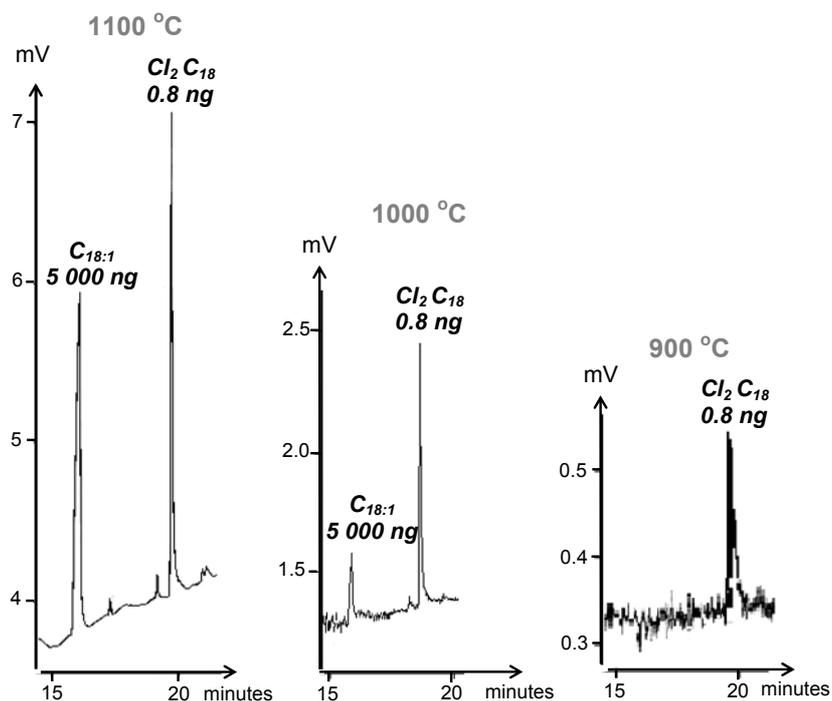


Figure 4. The XSD response of 0.8 ng  $\text{Cl}_2\text{C}_{18}$  methyl ester and 5 000 ng oleic acid ( $\text{C}_{18:1}$ ) methyl ester at reactor temperatures of 1100, 1000 and 900 °C. (Note the different scales of the y-axes).

#### *The stability of the XSD response*

The results obtained with the XSD were reproducible, which is important in trace analysis of  $\text{Cl}_2\text{FAs}$  in multiple samples. The responses of re-injected samples were almost the same even after 2-3 months between injections (Paper I), and the stabilisation time proved to be less than two hours, which is much shorter than that of the ELCD, which is about 1 day. Moreover, the ELCD is a more complex detector than the XSD. Both detectors operate with a mass transfer process over a gas-solid interface, but in the ELCD there is an additional mass transfer process over a gas-liquid interface. Less transfer processes and media in the XSD make this detector easier to handle, thus, suitable as a halogen selective detector for trace analysis of  $\text{Cl}_2\text{FAs}$  in multiple samples (Paper I and IV). The XSD is also cheaper to operate and maintain than the ELCD, as the latter is more labour intensive to operate.

## Isolation of chlorinated fatty acids

### Extraction of chlorinated fatty acids in lipids

The CIFAs in lipids have been extracted using various solvent mixtures such as acetone–cyclohexane (Vereskuns 1999), cyclohexane–propan-2-ol (Wesén *et al.* 1995a), hexane–acetone (Zhuang, Mc Kague & Reeve 2004) and chloroform–methanol (Björn 1999; Vereskuns 1999). To ascertain extraction of polar phospholipids (Zhukov & Vereshchagin 1981), the lipids in this project were extracted by chloroform–methanol according to (Bligh & Dyer 1959).

### Preparation of chlorinated fatty acid methyl esters

No single method or combination of methods can adequately prepare FAMES from all lipid classes (Kramer *et al.* 1997; Christie 2003). The method that best fits the circumstances must be chosen. Problems associated with the preparation of fatty acid esters include, incomplete conversion of lipids into FAMES, formation of artefacts, and residues of esterification reagents, which may damage the GC column (Shantha & Napolitano 1992). Common reagents for preparation of FAMES are base- or acid catalysed methylation agents and diazomethane, all with specific limitations. Base-catalysed reagents do not convert free fatty acids into FAMES, acid-catalysed reagents can destroy polyunsaturated fatty acids and cause artefacts, and diazomethane can react with double bonds and may explode, and its precursors are suspected carcinogens (Shantha & Napolitano 1992; Eder 1995). Some of the artefacts formed by acid-catalysed reagents can be counteracted by using freshly prepared reagents (Kramer *et al.* 1997; Christie 2003).

The acid-catalysed transesterification method, using sulphuric acid in methanol adapted from Christie (Christie 1989), is the most commonly used method for preparation of CIFAMES (Sundin *et al.* 1992; Wesén *et al.* 1992b; Mu *et al.* 1996a; Mu *et al.* 1996b; Björn *et al.* 1998). As multiple samples were analysed in this project, the rapid and simple transesterification method using borontrifluoride (BF<sub>3</sub>) (20%) in methanol (Morrison & Smith 1964) was used to prepare the CIFAMES. The preparation time of CIFAMES by using BF<sub>3</sub> in methanol is only about 30 min, compared to 12-16 hours for methanol containing sulphuric acid. However, although many reagents can be prepared whenever required BF<sub>3</sub> in methanol is best obtained from commercial sources, and an old reagent may generate artefacts. To confirm the results of the CIFAMES prepared by BF<sub>3</sub> in methanol some of the samples were therefore also prepared by using sulphuric acid in methanol adapted from Christie (1989).

### The importance of isolating the chlorinated fatty acids

The total concentration of EOCl in most fish samples ranges from 20 to 60 µg/g lipid (Lunde, Gether & Steinnes 1976; Håkansson *et al.* 1991; Wesén 1995) and, accordingly, the concentration of each species of CIFAs is a fraction of this. If Cl<sub>2</sub>C<sub>18</sub> and Cl<sub>2</sub>C<sub>14</sub> (the most commonly found CIFAs) contribute 15-20% each to the EOCl, their concentrations will correspond to 3-12 µg EOCl/g lipid and 15-60 µg Cl<sub>2</sub>C<sub>18</sub> or Cl<sub>2</sub>C<sub>14</sub>/g lipid in these fish samples. As the concentration of common

unchlorinated fatty acids can be  $\gg 1000$  times higher compared to the CIFAs the FAMES will overload the gas chromatograph capillary column when samples containing detectable amounts of CIFAMES are injected. Furthermore, common fatty acid derivatives have retention times overlapping the derivatives of several CIFA species (Figure 5), resulting in problems in the identification of the CIFAs. Therefore, to make possible the identification and quantification of CIFAs it is important to separate the CIFAMES and FAMES.

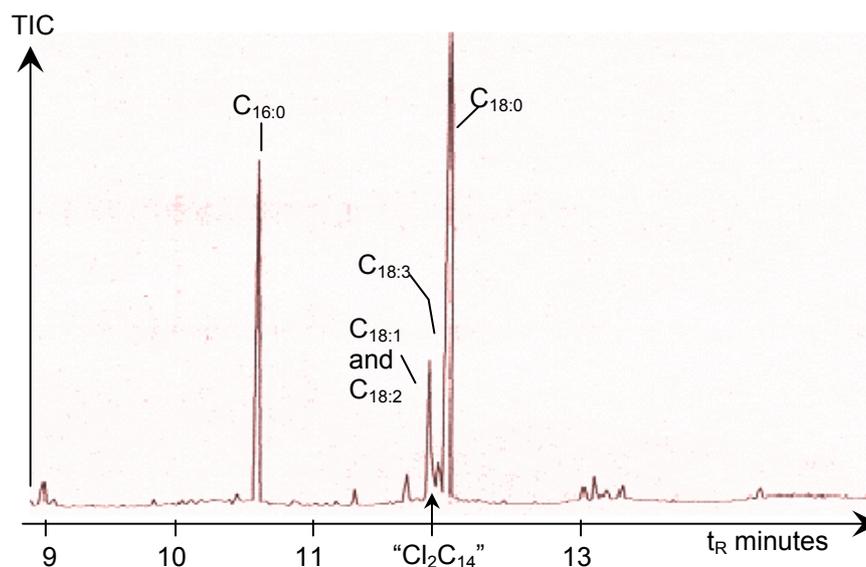


Figure 5. Total ion chromatogram (TIC) of methyl esters isolated from cell-cultured medium (containing the metabolite Cl<sub>2</sub>C<sub>14</sub>), before isolation of CIFAs by solid-phase extraction (SPE). The peak of Cl<sub>2</sub>C<sub>14</sub> is overlapped by derivatives of common unchlorinated fatty acids.

### Solid phase extraction in the isolation of chlorinated fatty acids

Solid phase extraction (SPE), which simply implies a physical extraction process involving a liquid and solid phase, has become a popular procedure for the separation of lipids and FAMES (Kaluzny *et al.* 1985; Zelles & Bai 1993; Ebeler & Ebeler 1996; Cripps & Tarling 1997), because it is simple and rapid. The packing material (the sorbent) is commonly held in a plastic tube by porous frits. In our case we used a silica based sorbent. To the silanol groups on silica surface, different types of organic groups can be bound, giving different types of selectivity to the sorbent. We used an amino-propyl silica as sorbent as it has been shown that different types of lipid classes can be separated efficiently on this type of sorbent using nonpolar solvents (Kaluzny *et al.* 1985). Using a polar stationary phase and a nonpolar eluent is called a straight phase procedure.

### *SPE in the separation of CIFAMEs and FAMEs*

In separation of different lipid classes by SPE using an amino-propyl column, as described by Kaluzny *et al.* (1985), *n*-hexane was used to collect the most unpolar neutral lipids such as fatty acid cholesterol esters (more polar lipids are retained on the column). The saturated and monounsaturated FAMEs are preferentially eluted with *n*-hexane because their interaction with the amino-propyl groups bound to the silica surface is very weak. By modifying this method, *e.g.* by increasing the volume of *n*-hexane it was possible to eliminate >99% of the saturated and monounsaturated FAMEs and >95% of all saturated and unsaturated FAMEs together from a mixture containing CIFAMEs (Paper II).

The separation of FAMEs and CIFAMEs is possible because the hydrophilic interaction between the CIFAMEs and the amino-propyl groups is stronger than that between the unchlorinated FAMEs and the amino-propyl groups. To disrupt the interaction between the CIFAMEs and the amino-propyl groups a more polar solvent must be used (Paper II). By using a solvent mixture of *n*-hexane–diethyl ether–dichloromethane; 89:1:10 (v/v/v), the CIFAMEs could be released from the column (Figure 6), and the recovery of the CIFAMEs was almost 100% in this fraction (Paper II). However, portions of the polyunsaturated FAMEs will still co-elute with the CIFAMEs (Figure 6), because double bonds are supposed to form stronger dipolar interactions with the stationary phase than is the case with saturated FAMEs (Wilson *et al.* 1993), resulting in a stronger retention of the mono- and polyunsaturated FAMEs. The polyunsaturated FAMEs require a more polar solvent such as dichloromethane to be quantitatively recovered (Wilson *et al.* 1993). As the CIFAMEs required an intermediately polar solvent mixture such as *n*-hexane containing a small portion of dichloromethane to be quantitatively recovered, some of the polyunsaturated FAMEs are suggested to be retained in the column. This possibly explains the low recovery of polyunsaturated FAMEs in the study of the separation of FAMEs and CIFAMEs (Paper II).

By collecting small fractions (1 ml) of the solvents it was possible to study the differential retention of the different unsaturated and polyunsaturated FAMEs. It was possible to separate the metabolite Cl<sub>2</sub>C<sub>14</sub> from most of the polyunsaturated FAMEs by collecting the second 1-ml fraction of the solvent mixture (Paper II). The SPE isolation procedure made it possible to remove the polyunsaturated FAMEs sufficiently to acquire a pure mass spectrum of the metabolite of Cl<sub>2</sub>C<sub>14</sub> as a methyl ester (Figure 7) and (Paper III). By collecting this 1-ml fraction it was also shown that the methyl esters of Cl<sub>2</sub>C<sub>14</sub>, Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>18</sub> have different retentions. Cl<sub>2</sub>C<sub>14</sub> and Cl<sub>2</sub>C<sub>16</sub> elute later than Cl<sub>2</sub>C<sub>18</sub> (Paper II), and this small differences in polarity between Cl<sub>2</sub>C<sub>18</sub>, Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>14</sub> as methyl esters might be sufficient for the development of a future method to isolate individual CIFAMEs by SPE.

The SPE procedure does not affect the composition of CIFAME species in an SPE treated eel lipid extract, in comparison with untreated extract, as is shown in Paper II. The XSD chromatograms obtained before and after SPE treatment showed an unchanged pattern of CIFAMEs (previously determined to be methyl

esters of saturated and unsaturated, dichlorinated fatty acids, as well as saturated tetrachlorinated fatty acids) (Paper II).

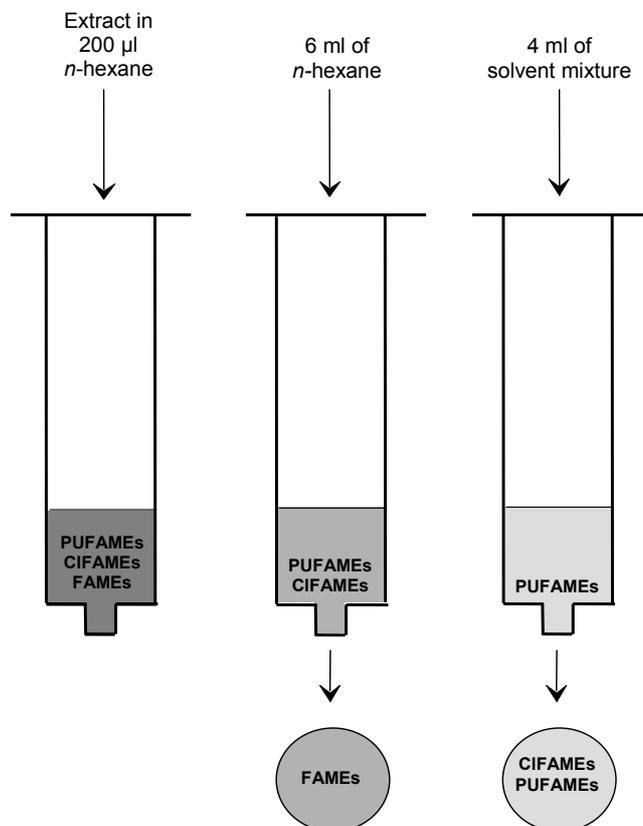


Figure 6. Solid-phase extraction in the separation of FAMES, CIFAMES and polyunsaturated fatty acid methyl esters (PUFAs). Solvent mixture of *n*-hexane–diethyl ether–dichloromethane; 89:1:10 (v/v/v).

#### *Removal of unknown compounds*

The SPE-procedure not only separates the CIFAMES from the FAMES, but also removes a bulk mass from the extract that either does not pass the GC column or generates a signal in the mass spectrometer. These compounds were calculated to contribute to about 75% of the total mass of the sample. By determining the gravimetric mass before and after SPE it was found that about 98% of the dry weight was removed.

The SPE procedure also made it possible to further isolate CIFAMES from an earlier silver nitrate and urea treated eel sample (Paper II). Most of the compounds in the eel extract that were applied to the SPE column were recovered in the *n*-hexane fraction, about 300  $\mu$ g of 340  $\mu$ g (dry weight), although the eel sample had been enriched 30-fold with respect to CIFAMES (Paper II). About 97-98% of the common FAMES were recovered in the *n*-hexane.

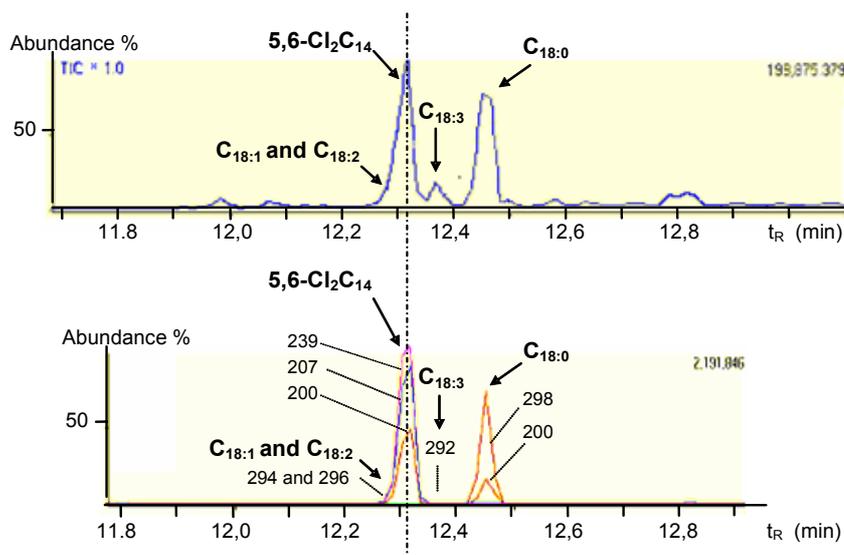


Figure 7. Total ion current (TIC) in GC/MS of the methyl ester of the metabolite 5,6-Cl<sub>2</sub>C<sub>14</sub> isolated by solid-phase extraction from the cell-culture medium (Paper IV). The lower panel shows the ion current (m/z) of selected ions characteristic of the methyl ester of the metabolite 5,6- Cl<sub>2</sub>C<sub>14</sub> and of closely eluting common fatty acids.

By assuming that <sup>i)</sup> only 2% of the compounds applied to the column are found in the fraction containing the CIFAMEs, that <sup>ii)</sup> the detection limit of CIFAMEs in the GC-XSD system is 0.5 ng, and that <sup>iii)</sup> a maximum of 5 µg can be injected into the GC-XSD system, the SPE procedure would make it possible to detect CIFAMEs at a level of about 1 µg/g of lipid (Paper II). This value of a CIFAME corresponds to about 0.2 µg EOC1/g lipid (the chlorine atoms contributes for about a fifth of the mass of a Cl<sub>2</sub>FA), which seems to be an appropriate concentration factor for detecting of CIFAs in human material.

The SPE procedure is preferable to the silver nitrate and urea complexation for the isolation of CIFAMEs. However, the residues of polyunsaturated FAMEs, which co-elute with the CIFAMEs, might be additionally eliminated by using complexation by silver nitrate. As the SPE procedure did not affect the composition of CIFAMEs species isolated from an earlier urea and silver nitrate treated eel sample, the treatment with silver nitrate should be appropriate to use before SPE.

## Identification of chlorinated fatty acids

The relative GC retention times can be used to identify ClFAMES species in fish samples (Mu *et al.* 1996a; Mu *et al.* 1996b; Zhuang *et al.* 2003a), but these data vary with operation parameters. Furthermore, different compounds may have the same retention time. By using MS more structural information of the ClFAs can be obtained and a mistaken identity of a ClFA can better be avoided. To obtain sufficient structural information of the ClFAs in EIMS analysis, the choice of ClFAs derivatives is important.

### Nitrogen-containing derivatives for mass spectral determination of chlorinated fatty acids

Nitrogen-containing derivatives have been found to exert a charge stabilisation effect upon carboxyl containing fragments of fatty acid derivatives produced using EI (Andersson & Holman 1974; Harvey 1982; Christie, Brechany & Holman 1987; Vetter, Meister & Oesterhelt 1988; Christie *et al.* 2000; Dobson & Christie 2002). These derivatives give simple mass spectrometric cleavage patterns from which the position of a double bond can be determined. Nitrogen-containing derivatives such as pyrrolidides and DMOX derivatives have been found to exert some stabilisation effects on Cl<sub>2</sub>FAs (Åkesson-Nilsson 1996; Zhuang, Mc Kague & Reeve 2004), but the EIMS fragmentation of the derivatives of the Cl<sub>2</sub>FAs is still too extensive. By selecting another nitrogen-containing derivative such as the picolinyl ester (Andersson & Holman 1974; Harvey 1982; Christie, Brechany & Holman 1987; Christie *et al.* 2000; Dobson & Christie 2002), that also can be used for the localisation of double bonds in unsaturated FAs, more structural information of Cl<sub>2</sub>FAs can be obtained (Paper III). In this study different species of Cl<sub>2</sub>FA, summarised in Table 1, were studied as picolinyl esters.

Table 1. Cl<sub>2</sub>FAs used as reference compounds in GC/MS analysis of the corresponding picolinyl esters, pyrrolidides and methyl esters.

Derivatives of	Cl <sub>2</sub> C <sub>14</sub> dichlorotetra- decanoic acid	Cl <sub>2</sub> C <sub>16</sub> dichlorohexa- decanoic acid	Cl <sub>2</sub> C <sub>18</sub> dichloroocta- decanoic acid	Cl <sub>2</sub> C <sub>20</sub> dichloroei- cosanoic acid	Cl <sub>2</sub> C <sub>22</sub> dichlorodo- cosanoic acid	Cl <sub>2</sub> C <sub>24</sub> dichlorotetra- cosanoic acid
Position of the Cl-atoms	9,10	9,10	6,7 9,10 11,12	5,6 11,12	13,14	15,16

### Picolinyl esters of chlorinated fatty acids

The picolinyl esters of Cl<sub>2</sub>FAs provided mass spectra, which were easy to interpret (Paper III). The principle to interpret the Cl<sub>2</sub>FAs as picolinyl esters is the same as for the corresponding monounsaturated FAs (Harvey 1982; Christie, Brechany & Holman 1987; Dobson & Christie 2002) as the chlorine atoms are eliminated when utilising MS in the EI mode, giving rise to unsaturation of the fatty acids. Intervals of 26 amu, instead of 28 units, between ions corresponding to dichlorinated fragments containing carbons *n-1* and *n+1* (Figure 8) were observed for Cl<sub>2</sub>FAs with the chlorine atoms positioned on carbons *n* and *n+1* (Figure 8). This agrees with the location of the double bond in a corresponding unsaturated FA moiety (an

interval of 26 amu locates a double bond between carbons  $n$  and  $n+1$ ) (Harvey 1982; Christie, Brechany & Holman 1987).

Another essential requirement for locating the position of chlorine atoms is the series of monochlorinated ions that are found for the picolinyl esters of  $\text{Cl}_2\text{FAs}$ . The monochlorinated ion with the lowest  $m/z$  value in this series is formed by cleavage between the carbon  $n+1$  (substituted with the second of the vicinal chlorine atoms) and carbon  $n+2$ . This ion is found in all mass spectra obtained from picolinyl esters of the  $\text{Cl}_2\text{FAs}$  and it can be predicted by the “198 + 14a” rule (Paper III). Here, 198 represents the mass of <sup>i)</sup> the  $\text{Cl}_2\text{FA}$  picolinyl ester including the carboxyl group (carbon 1) with an additional hydrogen atom, together with <sup>ii)</sup> the carbons  $n$  and  $n+1$  (where the chlorine atoms are suggested to be positioned) and 2 hydrogen atoms, and <sup>iii)</sup> a chlorine atom ( $198 = 137 + 26 + 35$ ). Further, the mass loss of 14 represents the mass of a methylene group and  $a$  represents the number of methylene groups between carbon 1 and carbon  $n$  (Figure 9). For example,  $a$  is 3 in 5,6- $\text{Cl}_2\text{C}_{20}$ ,  $a$  is 4 in 6,7- $\text{Cl}_2\text{C}_{18}$  and  $a$  is 7 in 9,10- $\text{Cl}_2\text{C}_{14}$ . A monochlorinated ion, essential for locating the position of chlorine atoms, is also present for DMOX derivatives of  $\text{Cl}_2\text{FAs}$ ; however, it is predicted by another rule (Zhuang, Mc Kague & Reeve 2004).

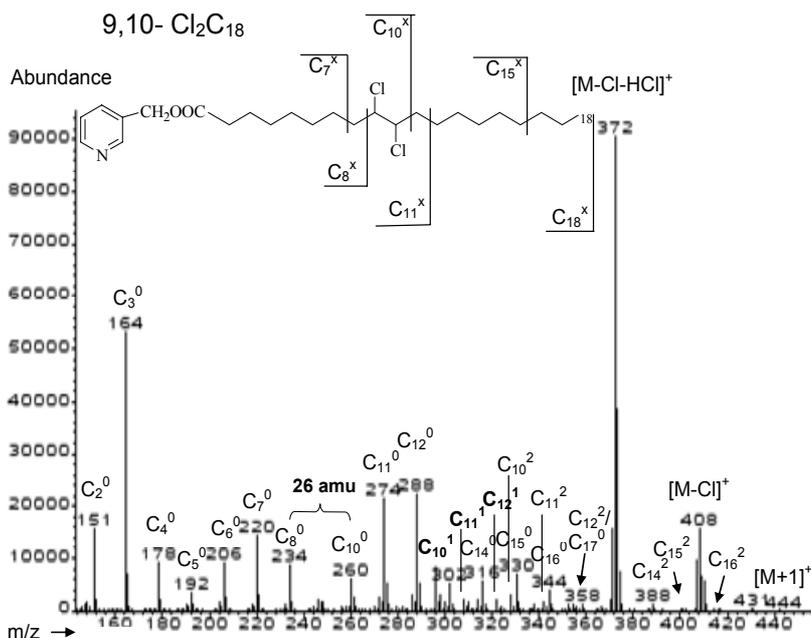


Figure 8. Mass spectrum of the picolinyl ester of 9,10-dichlorooctadecanoic acid.  $\text{C}_n^0$  = unchlorinated fragment,  $\text{C}_n^1$  = monochlorinated fragment,  $\text{C}_n^2$  = dichlorinated fragment.

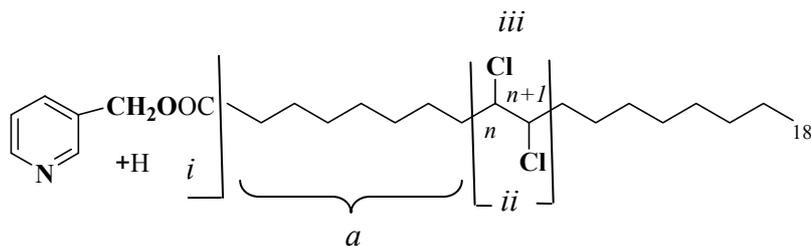


Figure 9. Picolinyl ester of 9,10-Cl<sub>2</sub>C<sub>18</sub> and an illustration of the “198 + 14a” rule. <sup>i)</sup> The picolinyl ester including the carboxyl group (carbon 1) with an additional hydrogen atom, <sup>ii)</sup> the carbons *n* and *n+1* and additionally 2 hydrogen atoms, and <sup>iii)</sup> a chlorine atom represents the total mass 137 + 26 + 35 = 198. *a* Represents the number of methylene groups between carbon 1 and carbon *n* and 14 represents the mass of the methylene group.

The prominent fragments corresponding to [M-2Cl-H]<sup>+</sup> and [M-Cl]<sup>+</sup> found in the high mass range of the picolinyl esters of Cl<sub>2</sub>FAs also give some structural information concerning the Cl<sub>2</sub>FAs and this can be useful when only small amounts of sample are available. The intensity of [M-2Cl-H]<sup>+</sup> is high and in some cases also the base peak, but the relative abundance of [M-Cl]<sup>+</sup> fragments increases as the chlorine atoms are positioned closer to the carboxyl group. For 5,6-Cl<sub>2</sub>C<sub>20</sub> the intensity of these fragments are almost the same as for [M-2Cl-H]<sup>+</sup> (Paper III) (Figure 10).

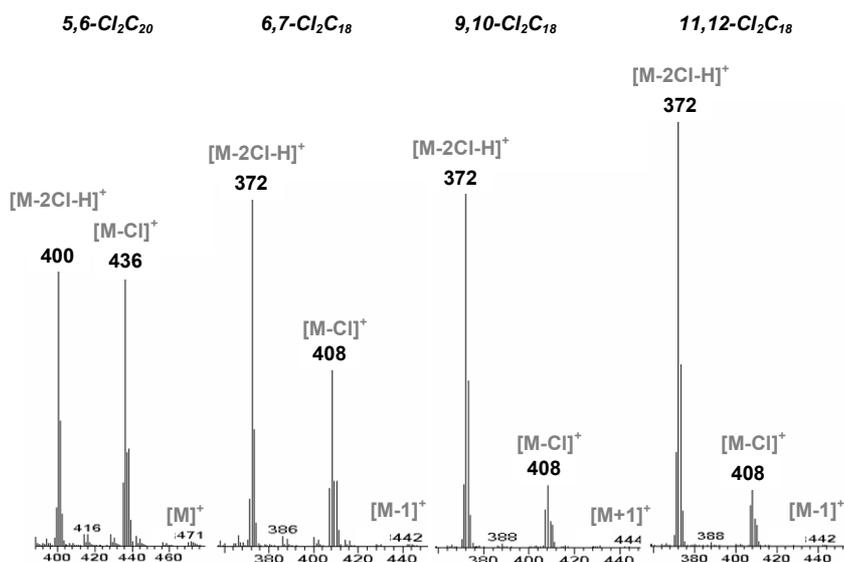


Figure 10. The relative abundance of [M-Cl]<sup>+</sup> fragments of picolinyl esters of chlorinated fatty acids increases as the chlorine atoms are positioned closer to the carboxyl group.

Even if the abundance of the monochlorinated fragments  $[M-Cl]^+$  is high, the intensity of the dichlorinated  $[M]^+$  ions is low and in many cases zero. However, by using the series of dichlorinated fragments, representing a loss of a methyl group and further losses of methylene groups, the molecular weight of the picolinyl esters can be indicated. The isotopic pattern shows that the fragments are dichlorinated (Paper III).

#### *Pyrrolidides of chlorinated fatty acids*

The pyrrolidides of  $Cl_2$ FAs (Figure 11) in EIMS analysis do not yield as good structural information as the picolinyl esters, but can serve as a complement to confirm information obtained from the picolinyl esters. The pyrrolidides are easier to prepare, as they can be prepared from FAMEs, whereas picolinyl esters only can be prepared from free fatty acids.

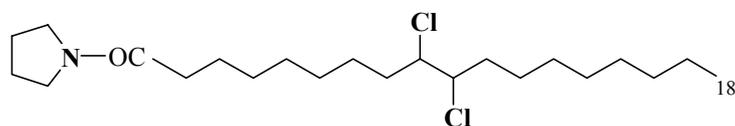


Figure 11. Pyrrolidide of 9,10- $Cl_2C_{18}$ .

In the study of  $Cl_2$ FAs summarised in Table 1 it was found that the position of the chlorine atoms can be determined approximately for the pyrrolidides, except for 6,7- $Cl_2C_{18}$ , in which their position could be determined exactly (Åkesson-Nilsson 1996; Paper III). In this mass spectrum an interval of 12 amu, instead of 14 units, between ions corresponding to fragments containing carbons  $n-1$  (C5) and  $n$  (C6) was observed for  $Cl_2$ FAs with the chlorine atoms positioned on carbons  $n$  and  $n+1$ . This corresponds with the localisation of a double bond between carbons  $n$  and  $n+1$  in a corresponding unsaturated fatty acid moiety (Andersson & Holman 1974; Christie, Brechany & Holman 1987). However, in pyrrolidides of  $Cl_2$ FAs with the chlorine atoms positioned further away from the carboxyl group the gap of 12 amu could appear somewhere between carbons  $n-1$  and  $n+2$ , which make the localisation more difficult to determine (Åkesson-Nilsson 1996). A series of monochlorinated fragments, corresponding to that used as an additional tool for the localisation of the chlorine atoms in the picolinyl esters and the DMOX derivatives of the  $Cl_2$ FAs, could not be obtained for the pyrrolidides. On the other hand, the absence of these monochlorinated fragments makes the mass spectra of the pyrrolidides less complicated compared to those of the picolinyl esters and DMOX derivatives of the  $Cl_2$ FAs. Furthermore, the abundant fragments corresponding to  $[M-Cl]^+$  make the pyrrolidides even more useful compared to the DMOX derivatives for the determination of molecular weight.

However, in some cases, *e.g.*, concerning the pyrrolidide of 5,6- $Cl_2C_{20}$ , the recovery was very low and the mass spectrum could not be studied. Probably one of the chlorine atoms was split off during the derivatisation, because two peaks with retention times corresponding to monochlorinated moieties were found in the

TIC and their fragmentation patterns matched, suggesting two structural isomers (data not shown).

### Methyl esters of chlorinated fatty acids

The methyl esters are not suitable for full characterisation of Cl<sub>2</sub>FAs, at least not for localisation of the positions of the chlorine atoms. However, the EI fragmentation pattern of 5,6-Cl<sub>2</sub>C<sub>20</sub> and 6,7-Cl<sub>2</sub>C<sub>18</sub>, with the chlorine atoms positioned close to the carboxyl group (Figure 12), showed distinct mass spectrometric fingerprints with specific ions related to the position of the chlorine atoms (Paper III). Corresponding fingerprints could not be obtained in the mass spectra of the other species of Cl<sub>2</sub>FAMES mentioned in Table 1.

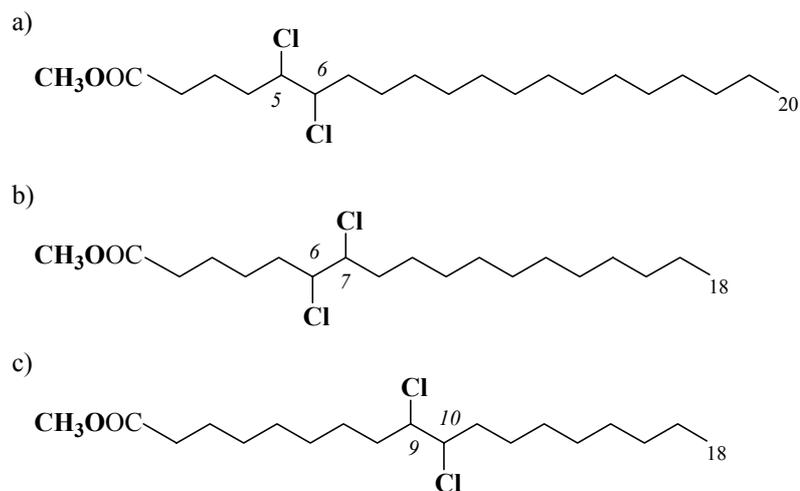
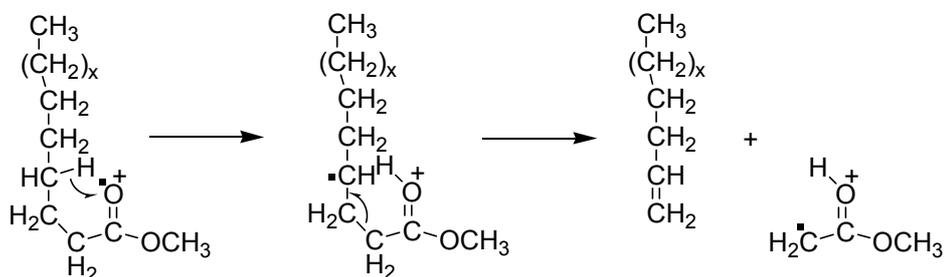


Figure 12. Methyl esters of a) 5,6-Cl<sub>2</sub>C<sub>20</sub> b) 6,7-Cl<sub>2</sub>C<sub>18</sub> and c) 9,10-Cl<sub>2</sub>C<sub>18</sub> fatty acids.

A stable monochlorinated fragment [M-74-HCl]<sup>+</sup> was only obtained for the Cl<sub>2</sub>FAMES with a chlorine atom positioned on carbon 6 (Paper III).

The characteristic McLafferty rearrangement ion at m/z = 74 (Figure 13) is normally the base peak for saturated FAMES (Christie, Brechany & Holman 1987) and Cl<sub>2</sub>FAs (Sundin *et al.* 1992; Mu *et al.* 1996a), but not for 5,6-Cl<sub>2</sub>C<sub>20</sub> (Paper III), which distinguishes position 6,7 from 5,6. The formation of the McLafferty rearrangement ion, which involves the hydrogen atom on carbon 4, might be impaired by an two electronegative chlorine atom positioned on carbon 5 (Figure 13).

a)



b)

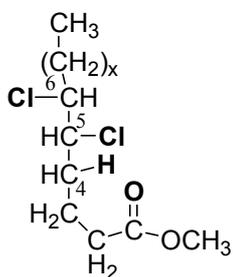


Figure 13. a) The formation of McLafferty rearrangement ion and b) an illustration of the involvement of the hydrogen atom on carbon 4 in the McLafferty rearrangement might be impaired by an electronegative chlorine atom positioned on carbon 5 in 5,6-Cl<sub>2</sub>C<sub>14</sub>.

## Determination of chlorinated fatty acids in cultured human cell-lines

### Incorporation of chlorinated fatty acids in human cell-lines

CIFAs are assimilated by biota with high efficiency from food and transferred in the food chain much in the same way as unchlorinated FAs (Cunningham & Lawrence 1976; Cunningham & Lawrence 1977a; Cunningham & Lawrence 1977b; Cunningham & Lawrence 1977c; Ewald *et al.* 1996; Björn 1999; Vereskuns 1999). To examine the possible incorporation of CIFAs into human cells, two different cell lines were used; Intestine 407 (INT 407), which is an epithelial cell line (Henle & Deinhardt 1957), and SH-SY5Y, a subclone of human bone marrow metastases (Biedler, Helson & Spengler 1973). The cells were cultured for 24 hours in media containing *threo*-9,10-Cl<sub>2</sub>C<sub>18</sub> and the cellular content of CIFAs was studied by extraction of the lipids, and transesterification to methyl esters followed by GC/XSD (Paper IV). The lipid class distribution of the CIFAs in the INT 407 cells was studied by separating different lipid classes (neutral lipids, free fatty acids and phospholipids) by using SPE and GC/XSD (Paper IV). The incorporation of CIFAs was also studied with a radiolabelled tracer, 1-<sup>14</sup>C-Cl<sub>2</sub>C<sub>18</sub>, followed by TLC with autoradiographic detection (Paper IV).

The study showed that human cell lines can incorporate extracellular 9,10-Cl<sub>2</sub>C<sub>18</sub> because Cl<sub>2</sub>FAs were found in both phospholipids and neutral lipids (Paper IV). The occurrence of Cl<sub>2</sub>FAs both in the phospholipids and the neutral lipids has also been observed in fish (Ewald *et al.* 1996; Björn *et al.* 1998a; Vereskuns 1999).

In the human cells, 9,10-Cl<sub>2</sub>C<sub>18</sub> was the predominating Cl<sub>2</sub>FA being incorporated, representing about 80% in both lipid fractions (Paper IV). This supports the earlier suggestion, that Cl<sub>2</sub>FAs remain intact during the incorporation, similar to the behaviour of unchlorinated FAs (Ewald *et al.* 1996; Björn 1999). However, the presence of minor amounts of Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>14</sub> in the phospholipids and neutral lipids suggests that 9,10-Cl<sub>2</sub>C<sub>18</sub> is metabolised to some extent by  $\beta$ -oxidation before being incorporated (Paper IV). Free Cl<sub>2</sub>FAs analysed as methyl esters were not detected, or were only present at very low levels compared to the amounts of ClFAs that could be released from neutral lipids and phospholipids (Paper IV). This is also the case for normal, unchlorinated fatty acids, which seldom occur free in living cells (Voet & Voet 1995).

The TLC study with autoradiographic detection showed that <sup>14</sup>C-labelled Cl<sub>2</sub>C<sub>18</sub> appears to be incorporated into the same phospholipids as <sup>14</sup>C-labelled stearic acid (C<sub>18:0</sub>), oleic acid (C<sub>18:1</sub>) and arachidonic acid (C<sub>20:4</sub>) (Paper IV), and this finding support previous indications (Ewald *et al.* 1996; Björn 1999) that ClFAs behave as normal fatty acids with respect to incorporation into the phospholipids.

### **Metabolism of chlorinated fatty acids in cultured human cell-lines**

ClFAs have been suggested to be degraded by  $\beta$ -oxidation (the fatty acid chain is degraded from the carboxyl end, by sequential removal of two carbon units to yield acetyl-CoA units for energy production; Kunau, Dommès & Schultz 1995; Stryer 1995; Voet & Voet 1995) (Conacher *et al.* 1984; Mu, Wesén & Sundin 1997; Björn 1999). Fatty acids in storage lipids can normally be metabolised completely by  $\beta$ -oxidation, but ClFAs seem to be degraded by  $\beta$ -oxidation only to a certain extent (Conacher *et al.* 1984; Mu, Wesén & Sundin 1997; Björn 1999), because Cl<sub>2</sub>C<sub>14</sub> is the shortest Cl<sub>2</sub>FAs with an even number carbon chain that has been reported. Perhaps  $\beta$ -oxidation of Cl<sub>2</sub>FAs is hindered by the bulkiness of halogen atoms which may obstruct the process when these are located too close to the carboxylic moiety of the molecule (Ewald & Sundin 1993). This probably explains why Cl<sub>2</sub>C<sub>14</sub> is one of the major ClFAs in aquatic animals from remote areas (Wesén *et al.* 1995a; Mu *et al.* 1996b; Milley *et al.* 1997; Björn *et al.* 1998; Zhuang *et al.* 2003a). However, the shortest ClFA that has been reported is Cl<sub>2</sub>C<sub>13</sub>, possibly formed by microbial metabolism (Björn *et al.* 1998), because microorganism can produce ClFAs with an odd number of carbon atoms from even-numbered chlorine-containing precursors (Murphy & Perry 1984, 1986).

The content of Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>14</sub> found in the lipids of the cultured cells after a 24-h incubation (Paper IV), suggests  $\beta$ -oxidation. However, in order to further study the metabolism of Cl<sub>2</sub>FAs in human cell-lines the cell-culture medium was

replaced by a new medium (not containing 9,10-Cl<sub>2</sub>C<sub>18</sub>) after 24 h of incubation, and it was then changed every fourth day. The content of the Cl<sub>2</sub>FAs in the cells and in the medium was determined every second day for up to 6 or 10 days (Paper IV).

Both cell-lines showed a time dependent decrease of 9,10-Cl<sub>2</sub>C<sub>18</sub> together with the parallel increase of Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>14</sub>. Metabolites of Cl<sub>2</sub>C<sub>18</sub> shorter than Cl<sub>2</sub>C<sub>14</sub> were not detected in the human cell-lines or their culture medium (Figure 14).

The shortest metabolite in the cultured INT-407 was identified as 5,6-Cl<sub>2</sub>C<sub>14</sub> (Paper III), which further supports the suggestion that Cl<sub>2</sub>C<sub>18</sub> is metabolised by  $\beta$ -oxidation (Paper IV). The identification of 5,6-Cl<sub>2</sub>C<sub>14</sub> isolated from fish downstream of a bleached craft mill (Zhuang *et al.* 2003a; Zhuang, Mc Kague & Reeve 2004) suggests the same. It is the position of the chlorine atoms on the carbons 5,6 that supports  $\beta$ -oxidation, because the chlorine atoms on carbons 9 and 10 on the carbon chain in Cl<sub>2</sub>C<sub>18</sub> will come 2 carbon atoms closer to the carboxyl group after every sequential removal of two carbon units. Nevertheless, it cannot be excluded that a small fraction of the Cl<sub>2</sub>C<sub>18</sub> may be metabolised through  $\omega$ -oxidation (oxidation of the carbon atom at the terminal, methyl end; Voet & Voet 1995; Bremer 2001). In a metabolic study of [18-<sup>14</sup>C]-9,10-Cl<sub>2</sub>C<sub>18</sub> in goldfish (*Carassius auratus*) low respiration of <sup>14</sup>CO<sub>2</sub> was found (Björn 1999), suggesting that part of the ClFAs might possibly be degraded by  $\omega$ -oxidation. Another option is that the ClFAs are dechlorinated and then degraded by normal  $\beta$ -oxidation.  $\omega$ -Oxidation is probably an unusual fatty acid oxidation pathway (Voet & Voet 1995), but highly substituted molecules have been found to be initially catabolised through  $\omega$ -oxidation (Gurr & Harwood 1996).

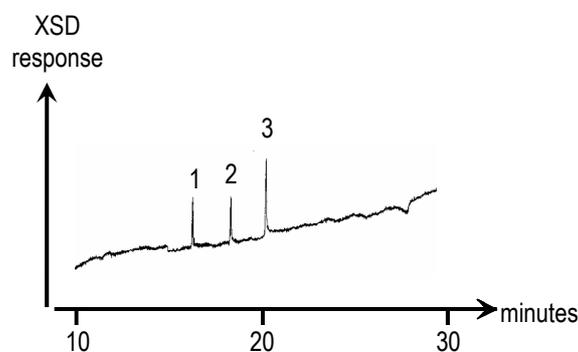


Figure 14. Cl<sub>2</sub>FAMES derived from Cl<sub>2</sub>FAs released from INT 407 cells (after 6 days of metabolism). The peaks correspond to the methyl esters of (1) 5,6-Cl<sub>2</sub>C<sub>14</sub> (2) Cl<sub>2</sub>C<sub>16</sub> (3) 9,10-Cl<sub>2</sub>C<sub>18</sub>. A BPX5 (unpolar capillary column) was used and the GC parameters were the same as for the analysis of Cl<sub>2</sub>FAMES in paper I. The XSD reactor temperature was 1100°C.

The relative distribution of Cl<sub>2</sub>FAs in the phospholipid fraction and the neutral lipid fraction of the INT 407 cells changed after further incubation (Paper IV).

Initially, 9,10-Cl<sub>2</sub>C<sub>18</sub> was the predominating Cl<sub>2</sub>FA in both membrane and storage lipids, but after 6 days, the amount of Cl<sub>2</sub>C<sub>16</sub> in the phospholipid fraction was significantly higher than the amounts of Cl<sub>2</sub>C<sub>18</sub> and Cl<sub>2</sub>C<sub>14</sub>. This is in contrast to the neutral lipid fraction, where the amount of Cl<sub>2</sub>C<sub>16</sub> was significantly lower than the amounts of Cl<sub>2</sub>C<sub>18</sub> and Cl<sub>2</sub>C<sub>14</sub> after 6 days (Paper IV). This might indicate that Cl<sub>2</sub>FAs bound in triacylglycerols have higher turnover rate than that of Cl<sub>2</sub>FAs bound in membranes, which is supported by earlier studies of fish lipids (Björn, 1999; Mu *et al.* 2004).

#### *Confirmation of $\beta$ -oxidation of chlorinated fatty acids*

By using SPE and picolinyl ester derivatives it was possible to isolate the metabolite Cl<sub>2</sub>C<sub>14</sub> (Figure 5) and determine the location of the chlorine atoms on the carbon chain. In the mass spectrum of the picolinyl esters of the metabolite Cl<sub>2</sub>C<sub>14</sub> an interval of 26 amu between the ions at  $m/z = 178$  and  $204$  (Paper III) was observed locating the chlorine atoms to carbons 5 and 6. The monochlorinated ion with the lowest  $m/z$  value in the series of monochlorinated ions was found at  $m/z = 240$  (Paper III), which according to the “198 + 14a” rule confirms the location of the chlorine atoms on carbon 5 and 6. Furthermore, the intensity of the fragments [M-2Cl-H]<sup>+</sup> and [M-Cl]<sup>+</sup> are almost the same, which also confirms that the Cl atoms are positioned closely to the carboxyl group (Paper III). This supports that Cl<sub>2</sub>FAs is degraded by  $\beta$ -oxidation.

By using the series of dichlorinated fragments in the high mass range, the molecular weight of the picolinyl ester of the metabolite was estimated to be 387 amu, which agrees with the calculated nominal molecular weight (Paper III). The [M]<sup>+</sup> ion was observed, albeit in low abundance.

To confirm the results obtained by the study of the picolinyl derivative the metabolite 5,6-Cl<sub>2</sub>C<sub>14</sub> was also studied as pyrrolidide derivative (Paper III). In the mass spectrum of the metabolite as pyrrolidides the base peak was found at  $m/z = 278$ , corresponding to [M-2Cl-H]<sup>+</sup>. Normally,  $m/z = 113$  is the base peak, but as the chlorine atoms are positioned closer to the carboxyl group the intensity of [M-2Cl-H]<sup>+</sup> and [M-Cl]<sup>+</sup> (at  $m/z = 314$ ) increases as found for the reference compounds. The molecular weight of the pyrrolidide of the metabolite was estimated to be 349 amu, which is in agreement with calculated nominal molecular weight, and confirms the molecular weight of the fatty acid.

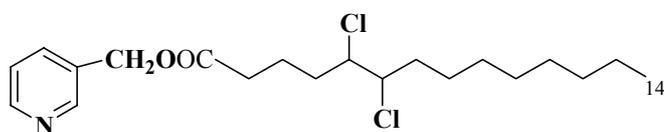
As expected, the position of the chlorine atoms in the metabolite as pyrrolidide derivative could only be determined approximately, because the 12 amu interval was observed between fragments which was the same as for 6,7-Cl<sub>2</sub>C<sub>18</sub> (Paper III). According to (Wolff & Christie 2002), a double bond positioned closer than position 7 from the carboxyl group does not produce the characteristic gap of 12 amu that can be used for location of the double bond. This might explain why the position of the chlorine atoms in the pyrrolidide metabolite derivative could not be determined exactly.

In the TIC of the pyrrolidides of 5,6-Cl<sub>2</sub>C<sub>14</sub> two peaks with retention times corresponding to monochlorinated moieties were also found, similar to the TIC of

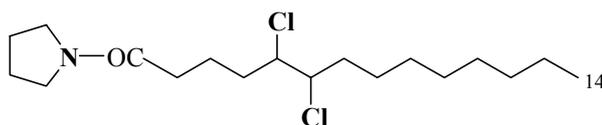
the pyrrolidides of 5,6-Cl<sub>2</sub>C<sub>20</sub>. Furthermore, the fragmentation pattern of these moieties were in agreement with the moieties of 5,6-Cl<sub>2</sub>C<sub>20</sub>, and the fatty acid carbon chain seemed thus to be chlorinated at the same carbon as in the moieties of 5,6-Cl<sub>2</sub>C<sub>20</sub> (data not shown).

The occurrence of the [M-74-HCl]<sup>+</sup> fragments (at m/z = 200 and 202), together with the fact that the McLafferty rearrangement ion at m/z = 74 was not the base peak in the mass spectrum of the methyl ester of the isolated metabolite, indicates that the chlorine atoms are positioned close to the carboxyl group, which agrees with 5,6-Cl<sub>2</sub>C<sub>14</sub>. The picolinyl esters, pyrrolidides and methyl esters of 5,6-Cl<sub>2</sub>C<sub>14</sub> are shown in Figure 15.

a)



b)



c)

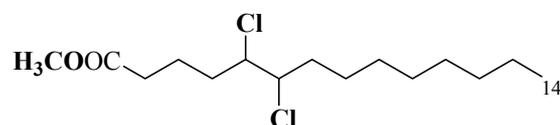


Figure 15. a) Picolinyl ester , b) pyrrolidide and c) methyl ester of 5,6-Cl<sub>2</sub>C<sub>14</sub>.

#### *Release of chlorinated fatty acids into the cell culture medium*

CIFAs do not induce elimination efforts in cells. Common ways to reduce the physiological impact of chemicals not being normal components of the organism (xenobiotics) include induction of the detoxifying hepatic enzyme system, cytochrome P450 (Alberts *et al.* 1994) and the activity of the hepatic enzyme EROD (7-ethoxyresurofin O-deethylase) (Stegeman *et al.* 2001). However, neither of these systems are activated by CIFAs (Håkansson *et al.* 1991; Goksøyr & Larsen 1993; Mu 1996). Therefore, an unexpected part of the study of the INT 407 cells and the SH-SY5Y cells was the observed release of incorporated Cl<sub>2</sub>FAs, in particular Cl<sub>2</sub>C<sub>14</sub>, into the culture medium (Paper IV) (Figure 16). However, the release of Cl<sub>2</sub>C<sub>14</sub> in both cell lines might suggest that the mechanisms responsible for the cellular removal of CIFAs is widely distributed among different human cell types, and possibly might be of general importance in the cellular management of xenobiotic fatty acids (Paper IV).

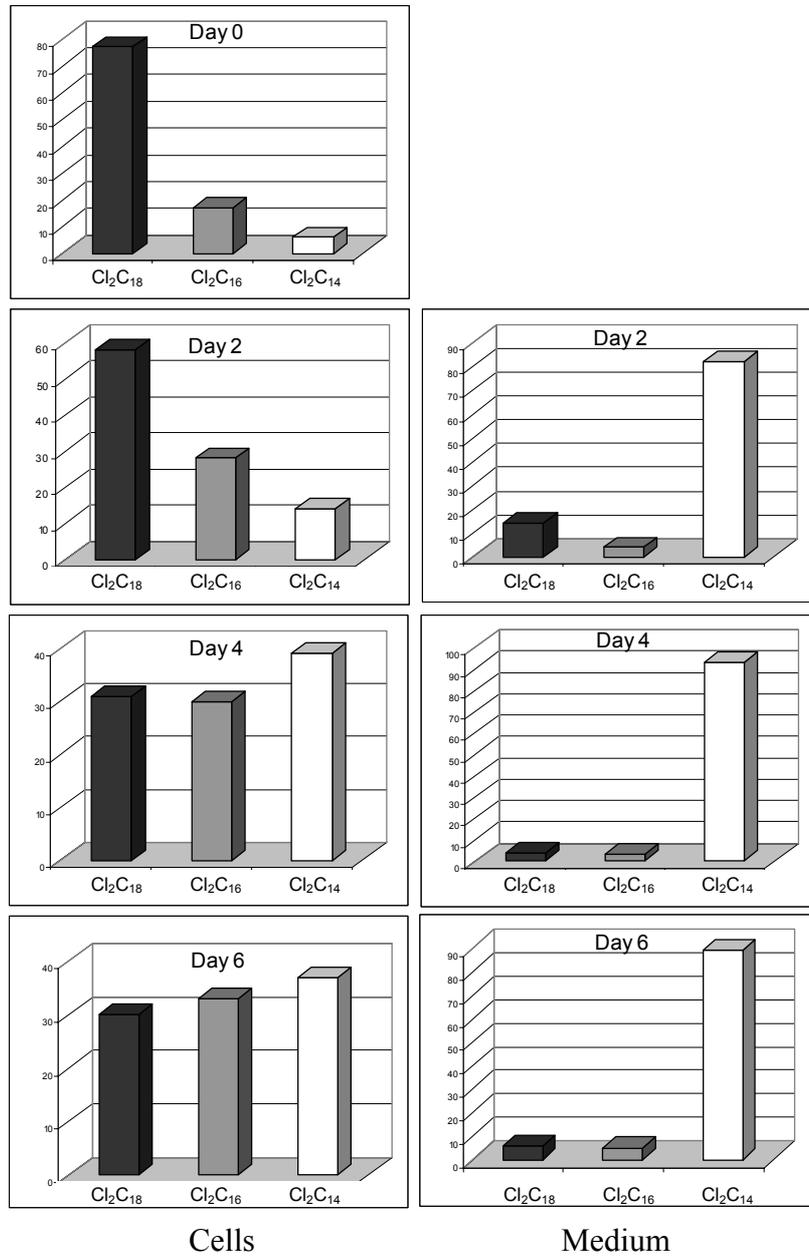


Figure 16. Relative distribution of Cl<sub>2</sub>C<sub>18</sub>, Cl<sub>2</sub>C<sub>16</sub> and Cl<sub>2</sub>C<sub>14</sub> in INT 407 cells from day 0 (after 24 hours incubation) to day 6 and in culture medium from day 2 to day 6. Culture medium was removed after 24 hours and after 4 days and new medium not containing Cl<sub>2</sub>FAs was added.

It seems reasonable to expect that the presence of Cl<sub>2</sub>FAs above a certain level in a cell might hamper normal cellular functions (Cherr *et al.* 1987; Håkansson *et al.* 1991; Björn, Sundin & Wesén 1998; Vereskuns 1999). The incorporation of ClFAs in membranes might possibly affect the maintenance of the normal cellular functions, on account of changes in the fatty acid profiles of membranes. The ratio between, *e.g.* saturated and unsaturated fatty acids in membrane phospholipids is an important factor in determining membrane fluidity and permeability (Stubbs & Smith 1984). The increased release of ATP observed in cells exposed to Cl<sub>2</sub>C<sub>18</sub> (Ewald & Sundin 1993), might be explained by the incorporation of ClFAs in the membrane.

## Conclusions

- The XSD is a good alternative to the ELCD in the determination of ClFAMEs released from complex samples. The XSD is stable, simple to handle and not too expensive to maintain.
- Human cells can incorporate extracellular *threo*-9,10-dichlorooctadecanoic acid, and degrade it to dichlorohexadecanoic acid and dichlorotetradecanoic acid. Dichlorotetradecanoic acid tends to be the shortest dichlorinated fatty acid formed, and is released to the culture medium.
- By using an aminopropyl solid-phase extraction column and picolinyl esters for mass spectrometric analysis it was possible to isolate and characterise 5,6-dichlorotetradecanoic acid, a metabolite from a cell-culture medium obtained by culturing human cell lines in media supplemented with *threo*-9,10-dichlorooctadecanoic acid. This provided evidence for that dichlorinated fatty acid Cl<sub>2</sub>FAs is initially degraded by  $\beta$ -oxidation, a fact that is useful in future studies of the origin of chlorinated fatty acids.

## Perspectives

In the present study, GC/XSD and SPE are described as useful methods for detection and isolation of Cl<sub>2</sub>FAs in cell-lines cultured in 9,10-Cl<sub>2</sub>C<sub>18</sub> and fish lipids. Furthermore, picolinyl esters of Cl<sub>2</sub>FAs in GC/MS analysis are a useful tool for the identification of metabolites of Cl<sub>2</sub>FAs obtained by the metabolism of Cl<sub>2</sub>FAs in the cultured cell cells. This may be of some importance in continued studies of the turnover of Cl<sub>2</sub>FAs in human cells, but also in future studies of chlorinated fatty acids in human tissue or in other environmental samples. Furthermore, the methods may also be of value in the determination of other halogenated fatty acids.

The use of a silver nitrate treated solid-phase extraction column before the amino-propyl column may make it possible to detect Cl<sub>2</sub>FAs metabolites that occur in very low concentrations. This may be a prerequisite in future research on the mechanisms underlying cellular functions related to Cl<sub>2</sub>FAs.

The human cell-lines INT-407 and SH-SY5Y were found to metabolise Cl<sub>2</sub>FAs by  $\beta$ -oxidation, and release 5,6-Cl<sub>2</sub>C<sub>14</sub> to a higher degree into culture medium compared to other Cl<sub>2</sub>FAs. This raises question about the cellular management of Cl<sub>2</sub>FAs, and the role of metabolism and in the regulation of cellular defence against xenobiotic fatty acids. Furthermore, the use of these cell-lines in combination with SPE isolation of metabolic products of Cl<sub>2</sub>FAs may aid in the determination of Cl<sub>2</sub>FAs distribution in phospholipids and neutral lipids, and more information of the metabolic pathways and physiological effects might be predicted.

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## Acknowledgements

The first part of this work has been carried out at the Department of Environmental Medicine Laboratory, Karlskrona, and the second part at the Department of Chemistry and Biomedical Sciences, Kalmar University.

This thesis would not have been completed without help and support from numerous people. I express my gratitude to all my colleagues at the Department of Chemistry and Biomedical Sciences for creating an inspired, pleasant and enjoyable working atmosphere. I am also grateful for my colleagues at the Department of Environmental Assessment, in Uppsala, for your support and the warm welcome I get every time I visit you.

I would also especially like to acknowledge the following people:

Kenth, my loving husband and life companion for supporting me, being patient, believing, and for loving me. You also made it possible for me to combine the family life and PhD-studies.

Emma and Oscar, my adorable children for being patient, for your love and for taking care of your challenging little brother Albin so many times for me.

Peter Sundin and Clas Wesén, my fantastic supervisors, who have taken part in my work since the beginning, for fruitful-co-operation, for guiding me through the field and never ending enthusiasm of my work and manuscripts, for having confidence and giving me total freedom in the lab, and also for being wonderful friends.

Ian Nicholls, an extraordinary supervisor during the second part of my work for volunteering to be my supervisor, even if your profession is not analysis of chlorinated fatty acids. Thank you for valuable criticism on my manuscripts and thesis, for helping me every time I knock on your door, and also for being a good friend and listener.

Henrik Kylin, my latest supervisor, for giving constructive criticism on my manuscripts and thesis, for your support, and for “gilting” the edge of my work.

Håkan Hallmer, my boss, for supporting and having confidence in me during the work. I must also thank you for giving me total freedom in planning my PhD-studies and my daily work at the department, and for valuable support when trying to combine work, PhD-studies and family life.

Peter Brodelius for having confidence, for keeping your GC/MS equipment in shape, and for giving me access to it in my work.

Jimmy Hedin, Mikael Nilsson, Sacha Legrand, Sarah Picaud and Per Mercke for sharing many GC/MS experiences and memories with me, and for providing me joyfulness during the work. I must also acknowledge Jimmy for not always being malicious.

Roland Isaksson for your engagement in improving my knowledge in separation techniques.

Jenny Rosengren-Holmberg, a trustable and generous colleague and friend. Thank's for always having your door open for me, my problems and when I'm thrilled. I must also thank you for being my lifeline when problems appear in the lab, which occasionally is the case.

Karina Adbo, a wonderful colleague and friend, for drinking black coffee (or whatever it is) and discuss the meaning of the life with me, "as adults". I also thank you for taking care of me when I "had" to stay over in Kalmar.

Susanne Wikman, a very nice, clever and efficient colleague, for once upon a time introducing me to GC/MS analysis and for providing joy and constructive criticism on my way of doing my work.

Katarina Gustafsson, my room ally, for believing and supporting me in my work. I also thank you for all nice discussions about everything and for cheering me up in the end of though days.

Kerstin Sandholm and Kristina Nilsson Ekdahl, for your engagement in future studies of my work. I also thank Kristina for helpful comments on my thesis.

People that also deserve extra attention, as they have contributed to the positive atmosphere at work are Annika Andersson, Linus Olofsson, Johan Svenson, Jesper Karlsson, Jonas Ankarloo, Pernilla Söderberg, Björn Karlsson, Nichole Kirsch and Per-Ola Andersson for providing me with hundreds of laughs during my time as PhD-student. Special thanks to Johan for proofreading my thesis.

Boel Lindegård, my dear general and analytical chemistry colleague, for reliving the pressure of "chemistry" teaching this semester.

Gleffe, Steffan, Bosse, Peter, Anette, Marlene and Eva for technical and lab. support. Lena, Lilita, Yvonne, Berit and Åsa for administration support.

*I would also acknowledge:*

Gina, Petra, Maria, Örjan, Marita, Christina H, Christina G. S., Helena B and Gastons V, who shared the first years with me, for being nice partners. I would especially like to thank Örjan for introducing me to the GC/MS equipment and helping me with the installation of the XSD detector and Christina Gustafson-Svärd for valuable support, fruitful co-operation, for introducing me to scientific thinking, and for providing the medium from cultures of human cell lines.

My mother, Inga-Lisa, for supporting me and for giving me much of your stubbornness, and never giving up attitude.

My father, Kjell, for teaching me to be curious, to never to stop questioning things, and to realize that the coin has more than one side.

My sisters and brothers, Elisabet and Margaretha, and Åke and Jan and for encouraging me and always being there. I must especially acknowledge Elisabet for being on duty every time I call you, for never ending positive attitude, and for sacrificing a lot for me and my family even if you have your own family, and even if takes more than two hours for you to reach us by car. I must also thank your husband Patrik. Thanks for giving me access to your wife.

The University of Kalmar, the Hospital of Blekinge and the Swedish University of Agricultural Sciences are acknowledged for financial support.